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Title: SEPARATION OF LYMPHOCYTES BY ELECTROPHORESIS
UNDER TERRESTRIAL CONDITIONS
AND AT ZERO GRAVITY

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Title:

Separation of Lymphocytes by Electrophoresis
Under Terrestrial Conditions and at
Zero Gravity

Phase II:

Summary 1/5/76 - 1/4/77

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PHASE II

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TWELVE MONTH SUMMARY REPORT (1/5/76 - 1/4/77)

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Peripheral blood mononuclear cells consist of several populations with distinct functions and distinct cell-surface properties. Cell surface structures have been used to identify some of these sub-groups -- for instance, T cells bear receptors for sheep red blood cells, B cells bear surface immunoglobulin, and complement receptor sites and B and some T cells bear receptors for the Fc portion of immunoglobulin. In addition, differences in the surface charge of T and B cells have also been reported. Electrophoretic mobility could offer a rapid and simple method for both quantitating and separating lymphocyte sub-populations. From a more fundamental point of view, it is important to determine precise cell-surface topography of lymphocytes and how these topographic structures relate to lymphocyte behavior in health and disease. The investigations have practical importance, in that it may be possible to separate sub-groups of lymphocytes that could be used therapeutically. "Suppressor" cells, for instance, may be important in prolonging organ grafts, and "Killer" cells could possibly be programmed to destroy tumors.

The first part of this report deals with studies on electrophoretic mobility of lymphocytes and the second with studies on lymphocyte activation and cell-surface topography.

PART I. ELECTROPHORETIC MOBILITY OF HUMAN PERIPHERAL LYMPHOCYTES

INTRODUCTION

Electrophoretic mobility (EPM) of human peripheral lymphocytes has been examined with the following objectives: To determine differences in EPM of lymphocytes under immuno-stimulated and immuno-suppressed states (1-4); To define the conditions necessary for the separation of lymphocyte sub-pop-

ulations in normal and pathological conditions (5-12); To investigate immunologically active, charged chemical groups on lymphocyte surfaces (5,13-17); and to investigate pathophysiological mechanisms of immune responsiveness (17,18), as reflected by alterations in EPM.

Patients challenged by a large organ graft, such as kidney, respond by developing various categories of plasma factors (antibodies) and cells (lymphocytes). Some of these factors are destructive to the graft (cytotoxic antibodies and killer lymphocytes) and others suppress the development of destructive antibodies and lymphocytes (enhancing antibodies and suppressor lymphocytes). The destruction of, or adaptation to, a graft depends on the equilibrium established between these competing events (19). If killer and suppressor lymphocytes have sufficiently different degrees of net electrical charge on their surfaces, different functional groups of lymphocytes may be distinguishable and separable by preparative cell electrophoresis. If this is the case, these electrokinetic properties of lymphocytes could have importance in both understanding of immunobiology and manipulation of immune system in diseases.

To evaluate the potential of lymphocyte electrophoresis as: 1) a means of monitoring the immune status of kidney transplant recipients, 2) in predicting the outcome of kidney transplants and 3) as a method for separation of lymphocyte subpopulations, we studied the EPM of unfractionated human peripheral lymphocytes and of populations enriched with T and "B" cells from normal adults, hemodialysis patients and kidney transplant recipients.

Materials and Methods

MATERIALS

The normal subjects' ages ranged from 20 to 43 years and included both sexes. No drugs of any kind were taken, for more than a week prior to study. The uremic subjects' ages ranged from 25 to 64 years and included both sexes. They had been on hemodialysis treatment (5 hours, thrice weekly) for at least one year and without any infection for at least one month prior to the study. All of these patients received multivitamins (one tablet/day), folic acid (1 mg/day), non-absorbable antacid, and some had been receiving dihydrotachysterol, ferrous sulfate and antihypertensive drugs. The transplant recipients' ages ranged from 24 to 56 years and also include both sexes. They had received renal allografts 3 days to 11 years prior to the study. Although none of these patients had infection, some of these had acute and/or chronic rejection, and all were receiving varying doses of prednisone and azathioprine at the time of the study. None received heterologous antilymphocyte globulin.

METHODS

Cell preparation

Lymphocyte separation: peripheral venous blood specimens were collected between 9 and 10 AM (one to 2 hours postprandial) from the study subjects. Twenty-five to 80 ml of freshly drawn venous blood was mixed with heparin (10 U/ml of blood) without preservatives (Panheparin[®], Abbotts Laboratory, North Chicago, IL.) layered on a Ficoll-Hypaque[®] mixture (Sp. gr. 1.078-1.080), and lymphocytes were separated after centrifugation (1,400 rpm, 40 min., 20°C). Separated lymphocytes were collected from the interface and washed three times with Ca and Mg free R₁'s balanced salt solution (HBSS,

Grand Island Biological Co., Grand Island, NY). To remove "adherent" cells (monocytes, neutrophilic leucocytes and B lymphocytes), the lymphocyte preparation was suspended in HBSS with Ca and Mg and 10% heat inactivated (decomplemented) fetal calf serum (FCS), and incubated on Falcon plastic petri dishes for 1 hour at 37°C in 95% O₂ - 5% CO₂. Decanted cells were re-suspended in HBSS and the number of viable lymphocytes counted in a hemocytometer using the Trypan blue dye exclusion test. This procedure usually provides over 90% pure and over 90% viable lymphocytes.

E rosetting procedure: Commercially (Flow Laboratory, Rockville, MD) prepared sheep red blood cells (SRBC) were washed twice in HBSS, centrifugated at 2,000 rpm for 5 min. at 4°C, and three drops of packed SRBC were suspended in 10 ml HBSS. Equal volumes of human lymphocytes [2×10^6 lymphocytes/ml of RPMI 1640 (Grand Island Biological Co., Grand Island, NY)] and 10% FCS and SRBC suspension were combined in Falcon plastic tubes, and centrifugated at 500 rpm for 5 min. at 4°C (20). The cells were incubated overnight (18 hours) at 4°C. The following day, pellets were gently re-suspended and cells with and without rosettes (lymphocytes with three or more of SRBC were considered as rosette-positive cells) were counted. The lymphocytes SRBC suspensions were layered over 12 ml Ficoll-Hypaque (Sp. gr. 1.078-1.080) and centrifugated at 1,400 rpm for 45 min. at 4°C. Non-rosetted lymphocytes were removed from the interface. The lymphocyte preparation which did not form rosettes with SRBC is referred to as non-T lymphocyte preparation and contains predominantly B lymphocytes, as well as null cells and a few monocytes. We will refer to these cells as "B" lymphocytes for convenience, recognizing that it is heterogenous with regard to cell type. Rosetted lymphocytes (T lymphocytes) in the pellet beneath the Ficoll-Hypaque were washed once with 0.83% ammonium chloride, 0.17 M Tris buffer,

pH 7.2, to lyse SRBC (21), collected by centrifugation (1000 rpm at 20°C for 15 min.), and washed twice with HBSS.

Lymphocyte electrophoresis

Electrophoresis (EP): Cells to be observed were washed twice with the electrophoretic solution of 0.03 mol liter⁻¹ NaCl, buffered to pH 7.40 ± 0.2 (280 ± 5 mOsm/Kg with 4.0 gm/100 ml of Sorbitol) and resuspended in the same solution at a concentration of 2 to 3 X 10⁶ cells/ml. EPM of lymphocytes was determined in a Cylindrical Microelectrophoresis Apparatus (Type U2, Rank Brothers, Bottisham, Cambridge, England). Both electrodes (platinum) and capillary chamber containing electrophoretic solution were kept at 25°C. The applied field strength was 3.22 V/cm (50 Volts and 3.3 mA).

Since EPM of erythrocytes and its stability have been established (22), fresh human erythrocytes obtained from healthy individuals were used as a reference material before and after each measurement of lymphocyte EPM to calibrate the apparatus. A small amount of the cell suspension was injected directly into capillary chamber with a microinjector consisting of 20 cm long polyethylene tubing (1 mm diameter) attached to 1 ml disposable syringe. The EPM was calculated from the time taken for the cells, in focus at "stationary layer" to traverse a distance of 25 or 37.5 µm (2 or 3 graticule squares) depending on the speed, so that the time measured was between 4 and 10 seconds. The polarity was reversed after each measurement so as not to polarize one electrode. Measurements were stored in the memory banks of HP-55 (Hewlett-Packard, model HP-55, Cupertino, CA), and recorded at the end of each sample study. The cell sample was used until all cells had sedimented from view, and exceptionally slow and deformed cells were excluded from the study. The procedure was repeated until 100 cells of a sample had been timed. Each cells EPM was expressed as units

defined in terms of velocity ($\mu\text{m}/\text{sec}$) and field strength (V/cm) as follows:

1 unit = $1 \mu\text{m sec}^{-1} \text{V}^{-1} \text{cm}^{-1}$.

Electrophoretic suspension media: Since optimal conditions (ionic strength and pH of the electrophoretic suspending media) of electrophoretic studies on human lymphocytes have not been previously systemically investigated, standardization of these conditions is a prerequisite to lymphocyte electrophoretic mobility measurements.

1. Ionic Strength of Suspension Media. The EPM of normal human lymphocytes was determined in electrophoretic suspension media of varying ionic strength (0.003, 0.006, 0.01, 0.03, 0.06, 0.10, 0.15 mol liter⁻¹ NaCl) while other factors were kept constant (isotonicity [$280 \pm 5 \text{ mOsm/kg}$] was maintained with varying concentration of Sorbitol [J. Baker Chemical Co., Phillipsburg, NJ], and pH maintained at 7.40 ± 0.2 with sodium bicarbonate, and temperature was kept at 25°C). This study showed that the EPM of lymphocytes was inversely proportional to the ionic strength of the electrophoretic suspension medium (Figure 1).

Since the mobility of lymphocytes increases markedly with decreasing ionic strength of the suspension medium, greater precision in the measurements can be achieved at lower ionic strengths. However, at very low ionic strengths macromolecules may adsorb more readily to the lymphocyte surface with resultant changes in electrophoretic behavior. Thus optimal conditions had to be established where the cells possess a high mobility but still display electrokinetic stability. We elected to use a lymphocyte suspending medium of 0.03 mol liter⁻¹ in subsequent studies, since this was low enough to measure cell mobility accurately and high enough to preclude artifactual changes in mobility arising from absorption of macromolecules to the peripheral zone of the lymphocytes.

2. pH of Suspension Media. Surface charge groups arise either by ionization of chemical groups or by adsorption of ions from the suspending medium. The pK of surface groups may be established from appropriate pH versus mobility relationships, where the pK of a surface group occurs at a pH value which corresponds to half the mobility obtained for a plateau value of mobility, i.e., a region where the mobility is independent of pH.

We determined the EPM of lymphocytes in media varying in pH from 3.0 to 11.0, while ionic strength was kept constant at either 0.03 or 0.15 mol liter⁻¹ NaCl. Again the influence of ionic strength on lymphocyte mobility was profound, but the pH did not have a significant effect on lymphocyte mobility, in the range between pH 6.0 and 9.0 (Figure 2). The mobility is markedly reduced in media of pH < 6.0. Thus, to avoid pH dependent effects on mobility, it was decided to fix the pH at 7.40 ± 0.2 in subsequent studies.

3. Reversibility of Electrophoretic Mobility of Lymphocytes. In separating lymphocytes from other blood components the cells are repeatedly exposed to solutions of varying ionic strength and chemical composition. Since ionic strength affects lymphocyte electrophoretic mobility, it was important to evaluate the effects of repeated exposure of lymphocytes to media with varying ionic strength on the final electrophoretic mobility.

Lymphocytes were suspended in a medium of 0.15 mol liter⁻¹ ionic strength at pH 7.4 ± 0.2 and then divided into several aliquots. The aliquots were then resuspended in media of varying ionic strength (0.003 - 0.15 mol liter⁻¹ NaCl) for one hour, and then returned to the original media (0.15 mol liter⁻¹ NaCl) and lymphocyte mobility was determined on each aliquot. Results of this experiment indicated that alterations in mobility induced by changes in ionic strength are reversed when the ionic strength is restored to its original value (Table 1). Exposure of lymphocytes to

the various solutions used is therefore unlikely to modify the ultimate results of lymphocyte electrophoretic mobility.

Subsequent to these experiments, all lymphocyte electrophoretic studies were conducted in a suspension medium of $0.03 \text{ mol liter}^{-1}$ NaCl ionic strength, buffered to $\text{pH } 7.40 \pm 0.2$ with sodium bicarbonate, at 25°C . The suspension media also contained $4.0 \text{ gm}/100 \text{ ml}$ of Sorbitol to maintain isotonicity ($280 \pm 5 \text{ mOsm/Kg}$).

Statistical analyses. Statistical significance of the differences observed between mean values of individuals or groups were evaluated by the critical ratio method (23).

Results

Electrophoretic Mobility of Peripheral Lymphocytes from Normal Adults, Hemodialysis Patients and Renal Transplant Recipients. EPM was determined on 13 lymphocyte specimens obtained from 7 healthy adults (4 males, 3 females; age range from 25 to 42 years). The results of this study are shown in Table 2. Range of EPM was -1.24 to -2.25 , and mean ($\pm \text{SE}$) was -1.79 ± 0.01 . Statistical analysis of these data indicates that there are no significant differences in mean EPM between male (1.78 ± 0.01) and female (1.80 ± 0.01), among individuals or in the same subjects with different specimens, sampled on different days.

Most of the lymphocyte EPM frequency polygons histograms obtained in this study appeared to have a bimodal distribution, suggesting the presence of at least two major and possibly several minor lymphocyte subpopulations (Figure 3). However, exact locations of the peaks in the histograms varied somewhat from individual to individual and even in the same individual sampled over a period of weeks. Thus, composite frequency polygons of

lymphocyte EPM from these subjects revealed less of a bimodal appearance and resulted in masking of the presence of lymphocyte subpopulations (Figure 4).

EPM of peripheral lymphocytes were serially determined from cadaveric kidney transplant recipients before and after transplantation (Short-term Study). This study included a total of 18 EPM determinations from 6 recipients, starting from 3 to 12 hours prior to transplantation (pre-immunosuppressive therapy) and repeated by 48 hours after transplantation, for a minimum of two, and a maximum of five times, at about one week intervals. This study includes 5 male and 1 female recipients, and their ages ranged from 27 to 50 years. All were receiving immunosuppressive therapy, consisting of 50 to 500 mg of prednisone and 100 to 200 mg of azathioprine per day at the time of the study. Among these, 3 recipients have functioning kidneys and 3 subsequently lost their kidneys due to irreversible rejection.

EPM of peripheral lymphocytes from 4 kidney transplant recipients (2 male and 2 female, whose ages ranged from 22 to 58 years) with well-functioning grafts from 1 to 3 years were also studied (Long-term Study). All of these patients were being treated with rather small fixed doses of prednisone (0 - 20 mg/day) and azathioprine (50 - 120 mg/day) for immunosuppression. No EPM studies were conducted on the lymphocytes of these latter patients prior to transplantation.

The results of these studies are shown in Table 3 (Short-term Study) and Table 4 (Long-term Study). The mean (\pm SE) EPM of lymphocytes in patients on maintenance hemodialysis treatment (-1.87 ± 0.04) determined immediately before transplantation was higher but not statistically significant than the mean EPM of normal lymphocytes (-1.79 ± 0.01). After transplantation, however, mean EPM of peripheral lymphocytes was significantly decreased (Mean \pm SE: -1.76 ± 0.02 , $p < 0.05$)

compared to the pre-transplant value (Mean \pm SE: -1.87 ± 0.04). Those patients who retained transplanted kidneys for 1 to 3 years, not only had a significantly ($p < 0.05$) lower mean EPM of their peripheral lymphocytes (Mean \pm SE: -1.68 ± 0.04) compared to hemodialysis patients but also when compared to that of the patients immediately post-transplant. However, there was no difference in mean EPM of peripheral lymphocytes between kidney transplant recipients whose grafts had functioned (Mean \pm SE: -1.78 ± 0.02) and those whose grafts had rejected (-1.74 ± 0.02).

Thus, EPM of peripheral lymphocytes in kidney transplant recipients is significantly lower than that of hemodialysis patients, regardless of doses of immunosuppressive drugs, duration of kidney transplantation, or presence or absence of rejection. Simple determination of EPM of peripheral lymphocytes in transplant recipients, however, does not provide any information on subsequent outcome of the grafts.

The EPM frequency polygons of peripheral lymphocytes in patients receiving hemodialysis treatment was distinctly different from normals and was characterized by a polymodal pattern, compared to the bimodal pattern seen in normals (pre-transplant frequency polygons in Figures 5 and 6).

After transplantation, the polymodal pattern of the frequency polygon of EPM changed further, usually within one week, into another pattern that was also polymodal, but characterized by increased proportions of lower mobility lymphocytes and decreased proportions of high mobility lymphocytes, associated with a decreased mean EPM (post-transplant frequency polygons in Figures 5 and 6). This increased proportion of lymphocytes with slow mobility is more prominent and consistent in kidney transplant recipients more than 1 year after the transplantation (post-transplant frequency polygon in Fig. 6) than in the recipients within 3 months after transplantation (post-transplant

frequency polygon in Fig. 5).

We have not detected differences in the evolution of EPM frequency polygons between those recipients who lost and those that kept their kidneys, nor were there specific patterns of evolution of the frequency polygons at different times following kidney transplantation in the same patients.

Electrophoretic Mobility of Peripheral T and "B" Lymphocytes in Normal Adults, Hemodialysis Patients and Kidney Transplant Recipients. EPM of T and B lymphocytes were studied in 5 normal adults, 5 uremic patients receiving chronic maintenance hemodialysis and 15 kidney transplant recipients.

The normal subjects' ages ranged from 20 to 40 years, and 3 were males and 2 females. The uremic subjects' ages ranged from 25 to 64 years and all these were males and had been stable on hemodialysis for at least one year prior to the study. Ages of the transplant recipients ranged from 24 to 56 years, and all but three of these recipients were male. Seven of the patients received renal allografts 3 to 28 days prior to this experiment (Short-term Study), and were on immunosuppressive therapy consisting of 50 to 200 mg of prednisone and 100 to 200 mg of azathioprine per day. The remaining 8 transplant recipients received renal allografts 8 months to 11 years prior to this experiment (Long-term Study), and were also on immunosuppressive drugs but were receiving lower doses of prednisone (10 to 20 mg/day) and azathioprine (100 mg/day). Although all the patients in the Long-term Study group had good and stable graft function (serum creatinine \leq 2.0 mg/100 ml), 3 of the 7 patients in the Short-term Study group had no significant graft function at the time of the study and 2 of these patients subsequently lost their transplants due to irreversible graft rejection.

Results of these studies are shown in Table 5 and in Figures 7 to 12. Although the mean EPM of T and "B" lymphocytes were identical in normal

adults, the mean EPM of "B" lymphocytes were significantly lower ($p < 0.003$) than that of T lymphocytes in both dialysis patients and transplant recipients. Furthermore, EPM of T and "B" lymphocytes of both dialysis patients and transplant recipients were also significantly lower ($p < 0.003$) than that of normals, and the EPM of "B" lymphocytes in transplant recipients exhibits the lowest mobility. Mean EPM of either T and "B" lymphocytes of the immediate post-transplant patients (Short-term Study group) were, however, similar to that of patients several months to several years post-transplant (Long-term Study group). Thus, EPM of both T and "B" lymphocytes are significantly decreased in hemodialysis patients and kidney transplant recipients. However, the slowest EPM is most consistently observed in "B" lymphocytes from transplant recipients, but the degree of slowness correlated poorly with duration of transplantation, doses of immunosuppressive drugs, degree and stability of the kidney function or eventual outcome of the transplanted kidney.

Composite frequency polygons of T and "B" lymphocytes studied in all three groups appeared to have Gaussian distribution with minimum skewness to either high or lower mobility side, suggesting that both T and "B" lymphocyte subpopulations were electrophoretically relatively homogenous cell populations. Despite the significant differences between the mean EPM of T and "B" lymphocytes in both hemodialysis patients and transplant recipients, major portions of EPM frequency polygons were superimposed on each other in both groups of patients. Thus, it appears that T and "B" lymphocyte subpopulations are difficult to identify or separate from one another as homogenous cell populations by means of lymphocyte electrophoresis, except for the small fractions of cells in areas of either extreme high or low mobility. Modification, however, of the net surface electrical charge of lymphocytes, by chemical or immunological treatment, may allow such separation.

Effects of Methylprednisolone and Transplant Recipients' Sera on Electrophoretic Mobility of B Lymphocytes. Lymphocytes were obtained from 5 normal adults (3 males and 2 females with age range from 27 to 38 years). Each subject's "B" lymphocytes, separated by the method described previously, were divided into three portions and were suspended in HBSS containing either 20% donor's serum, 20% donor's serum with 10 µg/ml of succinyl methylprednisolone (Solu-Medrol[®], Upjohn Co., Kalamazoo, Mich.), or 20% transplant recipient's serum at a cell concentration of 10×10^6 /ml, and were incubated at 37°C in 95% O₂ - 5% CO₂ for 18 hours. At the end of incubation, the "B" lymphocytes were washed twice with HBSS followed by two more washes with electrophoretic suspension media. The sera used in this experiment had been obtained from 5 transplant recipients on the same day we had studied the EPM of their T and "B" lymphocytes that were shown to have reduced mobilities.

The results of this study are shown in Table 6. EPM of "B" lymphocytes were not affected by incubation with methylprednisolone at a concentration of 10 µg/ml for 18 hours incubation. Although in vivo effects of methylprednisolone on the EPM of "B" lymphocytes might be different, it appears that the observed slow EPM of "B" lymphocytes in transplant recipients was probably not due to circulating methylprednisolone. Contrary to methylprednisolone, EPM of "B" lymphocytes was rather increased when the cells were incubated in 20% transplant recipients sera for 18 hours. Thus, decreased EPM of "B" lymphocytes observed in transplant recipients was evidently not caused by any humoral factors, either immunological or pharmacological, present in the recipients' sera.

Effects of Normal Sera on Electrophoretic Mobility of B Lymphocytes from Kidney Transplant Recipients. At this time, "B" lymphocytes were

obtained from two transplant recipients (25 and 32 year old males, 3 weeks and 3 months post-transplant, respectively). Each recipient's "B" lymphocytes were divided into two portions and were suspended in HBSS containing 20% of either the patient's own serum or freshly prepared normal human AB serum, and incubated for 18 hours at 37°C in 95% O₂ - 5% CO₂. At the end of incubation, the "B" lymphocytes were washed twice with HBSS followed by two more washes with electrophoretic suspension media.

The results of this study are shown in Table 7. Although the number of cases studied in this experiment is small, the trend of the results is that decreased EPM of "B" lymphocytes from transplant recipients was not reversed by incubation of the lymphocytes in normal human sera. Thus it seems that the alteration of surface electrical densities in "B" lymphocytes observed in the transplant recipients is an irreversible intrinsic phenomenon of the cells rather than a transient alteration of the cell surface due to extracellular factors.

Discussion

End-stage kidney disease is unfortunately a common, often fatal disease, and it claims about a half-million lives every year in the U.S. alone (24). The only available treatments for end-stage kidney disease, chronic dialysis and kidney transplantation have been extensively studied, experimentally and clinically for the past two decades. Although dialysis treatment can prolong lives of these patients, many of the patients have chosen kidney transplantation as the ultimate treatment for the disease since only a successful kidney transplantation can cure the basic disease. As a result, more than 8,000 kidney transplantations had been performed as of 1974 in the U.S., and it

has been projected that at least 3,500 kidney transplantations will be performed each year in the following years (25). Unfortunately, however, results of graft survival at 2 years have remained in the range of 40% - 85% depending on the kidney donor (26, 27), and, according to Terasaki's recent findings, the survival rate of cadaveric kidney transplants is actually decreasing at a rate of 2% per year (28). Among other factors, the immunologic barrier and transplant rejection have been the major cause of kidney transplant failure; more than 60% in general (26) and over 80% in some recipients of cadaveric donor grafts have failed (27).

Recognition of foreignness and activation of the immune response is a function of receptors on the surface of the lymphocytes. Each specific group of lymphocytes, whether classified morphologically or functionally has specific receptors consisting of surface chemical groups. Since EPM of lymphocytes is a measure of the Zeta potential of the cells, the EPM depends on the net electric charge density, which is negative and arises from α -carboxyl groups of N-acetyl neuraminic acid, phosphate groups or protein carboxyl groups (14). Some positive charge groups such as amino groups also exist at the lymphocyte surface. Thus, measurements of EPM may be a useful means to study surface properties of the lymphocytes.

Several investigators have attempted to distinguish subpopulations of normal human peripheral blood lymphocytes by means of EPM, and the studies have shown the presence of an essentially unimodal mobility distribution (9, 10), despite considerable differences in mobilities between T and B lymphocytes (8, 29, 30). This is contrary to repeated observations of a bimodal distribution of lymphocyte mobility in mice (6, 31 - 33). Our study, however, suggests that there are at least two electrophoretically distinguishable

subpopulations in normal human peripheral lymphocytes, although electrophoretic properties of the two populations overlap considerably. Since the ranges and means of pooled T and "B" lymphocytes' EPM data in this study are identical, bimodal distribution of peripheral lymphocyte mobility is unlikely due to different EPM distribution of T and "B" lymphocytes.* As Vassar et al (9) pointed out, however, there is considerable variability in distribution of lymphocyte mobility from individual to individual and even in the same subject sampled periodically over the course of several weeks, variability in the mean EPM remains small. Therefore, composite frequency polygons pooled from many subjects conceal the bimodal distribution of individual EPM of peripheral lymphocytes, thus indicating that pooling of electrophoretic data can be misleading.

Individual frequency polygons of lymphocyte EPM from chronic hemodialysis patients and kidney transplant recipients are distinctly different from normal and are characterized by a polymodal distribution. EPM frequency polygons from transplant recipients are further altered from normals and hemodialysis patients because of an increased proportion of slow mobility lymphocytes. Here again, individual frequency polygons vary so widely that composite studies of lymphocyte mobility from these patients completely conceals the distribution seen in individual cases. As shown in subsequent studies, the increased proportion of slow mobility lymphocytes primarily consisted of "B" lymphocytes with T lymphocytes making a rather small contribution to it. The increased proportion of slow "B" lymphocytes is most evident in the transplant recipients.

Since decreased electrophoretic mobility of "B" lymphocytes is uniformly observed in all transplant recipients, regardless of the duration of renal

*Heparin and ammonium chloride-tris buffer used in this experiment will not affect the EPM of the lymphocytes (40). However, it is not certain whether or not E rosette formation will alter EPM of T lymphocytes after SRBC are removed.

transplantation, doses of immunosuppressive drugs and the degree of graft function, this change is probably not due to surgical stress, immunosuppressive drugs or uremic toxins. Thus, it appears that decreased mobility of B lymphocytes in these patients reflects actual alterations of B lymphocyte subpopulations or alterations in functional status (thus modifying net electrical charge of lymphocyte surface) of existing B lymphocyte subpopulations as a response to kidney transplantation per se. This view is further supported by the observations that the EPM of "B" lymphocytes from normal subjects is not decreased by incubating them in transplant recipients' sera with or without methylprednisolone and the EPM of "B" lymphocytes from transplant recipients is not normalized by incubating them in normal human sera. Although Bert et al (2) have shown that prednisolone can reduce EPM of peripheral lymphocytes in vitro, the concentration of the drug they used (100 µg/ml) is far beyond pharmacologic levels and is 10 times higher than ours (10 µg/ml). The level we used is an equivalent serum concentration of the drug to that found 2 hours after 1,000 mg intravenous administration in adults (34). Furthermore, none of the transplant recipients we studied ever have received more than 500 mg of prednisolone per day. Thus these experiments rule out any direct effects of methyl prednisolone or other humoral factors in the transplant recipients as a cause of the decreased "B" lymphocyte EPM in the patients, although alterations in subpopulations could certainly result from the in vivo effects of immunosuppressive drugs.

Decreased EPM of lymphocytes has been observed in lymphocytes treated with anti-lymphocyte globulin (1,2,35,36), prednisolone (1,2), and asparaginase (37), in immuno-stimulated lymphocytes [treated with PHA (1),

as purified protein derivatives (1,15,22)], in anti-immunoglobulin antibody treated lymphocytes (14,18,38), enzyme treated lymphocytes [neuraminidase (13, 16)] and in certain diseases [rheumatoid arthritis (12)]. Thus, reduction in EPM of lymphocytes is a non-specific phenomenon common to several immunologic and non-immunologic manipulations. Since, according to the clonal theory (39), an entirely new subpopulation of lymphocytes cannot be produced by kidney transplantation, a series of studies suggests that decreased EPM of "B" lymphocytes in kidney transplant recipients is due to either rapid proliferation of existing small numbers of "B" lymphocytes with low net negative electrical densities or alterations in functional status, which influences the net electrical density on the surface of existing "B" lymphocyte subpopulation as a response to either kidney transplantation or immunosuppressive therapy. However, morphological and functional characteristics of the "B" lymphocyte subpopulation with very low EPM in transplant recipients remain to be clarified. A preparatory cell electrophoresis could separate this specific subset of "B" lymphocytes with very low EPM from the rest of "B" lymphocytes as well as T lymphocytes, and should provide an opportunity to study the immunobiological nature of these cells.

Since we have not observed any differences in EPM of either peripheral lymphocytes or peripheral T and "B" lymphocyte subpopulations between those who kept transplanted kidneys and those who rejected kidneys, simple determination of lymphocyte EPM does not provide prognostic information on either immunological interrelationship between the host and the graft (rejection vs. adaptation) or eventual fate of the graft.

Conclusions

Electrophoretic mobility of peripheral lymphocytes from normal adults, chronic maintenance hemodialysis patients and kidney transplant recipients at various clinical stages have been measured using a Cylindrical Micro-electrophoresis Apparatus, and the following conclusions have been drawn from the results of this study:

a. Electrophoretic mobility of peripheral lymphocytes from normal subjects show a bimodal distribution, suggesting the presence of at least two electrophoretically distinguishable lymphocyte subpopulations in peripheral blood.

b. Electrophoretic mobility of "B" lymphocytes from hemodialysis patients and kidney transplant recipients is significantly decreased from normal, and transplant recipients exhibit the greatest change.

c. A series of experiments suggest that decreased electrophoretic mobility of "B" lymphocytes in kidney transplant recipients is due to actual alterations in proportions of subsets of "B" lymphocytes and/or alterations in functional status of existing "B" lymphocyte subsets as a response to kidney transplantation.

d. Electrophoretic mobility of lymphocytes correlated poorly with duration of transplantation, doses of immunosuppressive drugs, presence or absence of rejection episode or eventual outcome of the transplanted kidney.

e. Electrophoretic technique can be used to identify and separate a subset of "B" lymphocytes with very low electrophoretic mobility from both T and "B" lymphocytes with higher electrophoretic mobility in kidney transplant recipients.

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LEGENDS TO FIGURES

- Figure 1. Electrophoretic mobility of peripheral lymphocytes from normal subjects in varying ionic strengths of suspension media.
- Figure 2. Electrophoretic mobility of peripheral lymphocytes from normal subjects in varying pH and ionic strengths of suspension media.
- Figure 3. A representative electrophoretic mobility of peripheral lymphocytes from a normal subject (case No. 3 in Table 2).
- Figure 4. A composite frequency polygon of electrophoretic mobility of peripheral lymphocytes from 7 normal subjects.
- Figure 5. Electrophoretic mobility of peripheral lymphocytes from a patient pre and post kidney transplantation (case No. 4 in Table 3).
- Figure 6. Electrophoretic mobility of peripheral lymphocytes from a patient pre and post kidney transplantation (case No. 5 in Table 3).
- Figure 7. Electrophoretic mobilities of peripheral T and "B" lymphocytes from 5 normal subjects.
- Figure 8. Electrophoretic mobilities of peripheral T and "B" lymphocytes from 5 hemodialysis patients.
- Figure 9. Electrophoretic mobilities of peripheral T and "B" lymphocytes from 17 kidney transplant recipients.
- Figure 10. Comparison of electrophoretic mobilities of peripheral T lymphocytes among normal subjects, hemodialysis patients, and kidney transplant recipients.

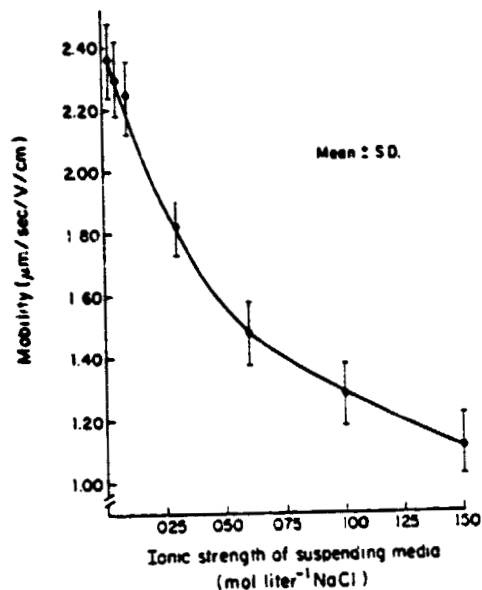
Figure 11. Comparison of electrophoretic mobilities of peripheral "B" lymphocytes among normal subjects, hemodialysis patients, and kidney transplant recipients.

Figure 12. Comparison of electrophoretic mobilities of peripheral "B" lymphocytes of kidney transplant recipients between short-term and long-term follow-up groups.

TABLES

- Table 1. Reversibility of electrophoretic mobility of lymphocytes in different ionic strengths.
- Table 2. Electrophoretic mobility of peripheral lymphocytes from normal subjects.
- Table 3. Electrophoretic mobilities of peripheral lymphocytes from hemodialysis patients and kidney transplant recipients.
- Table 4. Electrophoretic mobility of peripheral lymphocytes from transplant recipients (long-term study).
- Table 5. Electrophoretic mobilities of T and "B" lymphocytes from normal subjects, hemodialysis patients, and kidney transplant recipients.
- Table 6. Effects of methylprednisolone and transplant recipient's serum on electrophoretic mobility of "B" lymphocytes from normal subjects.
- Table 7. Effects of normal serum on electrophoretic mobility of "B" lymphocytes from transplant recipients.

ELECTROPHORETIC MOBILITY OF LYMPHOCYTES IN VARYING STRENGTH OF SUSPENDING MEDIA



REPRODUCIBILITY OF THE ORIGINAL PAGE IS POOR

Figure 1

ELECTROPHORETIC MOBILITY OF LYMPHOCYTES IN VARYING pH OF SUSPENDING MEDIA

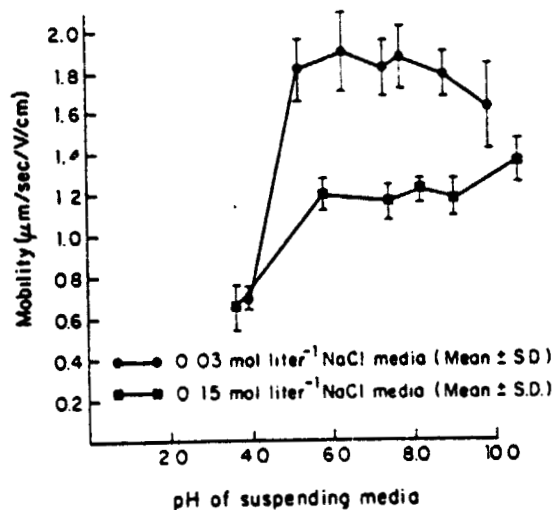


Figure 2

NORMAL SUBJECT

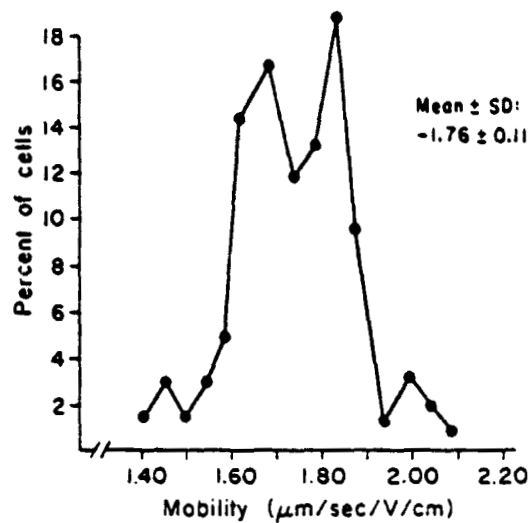


Figure 3

COMPOSITE HISTOGRAM OF NORMAL SUBJECTS

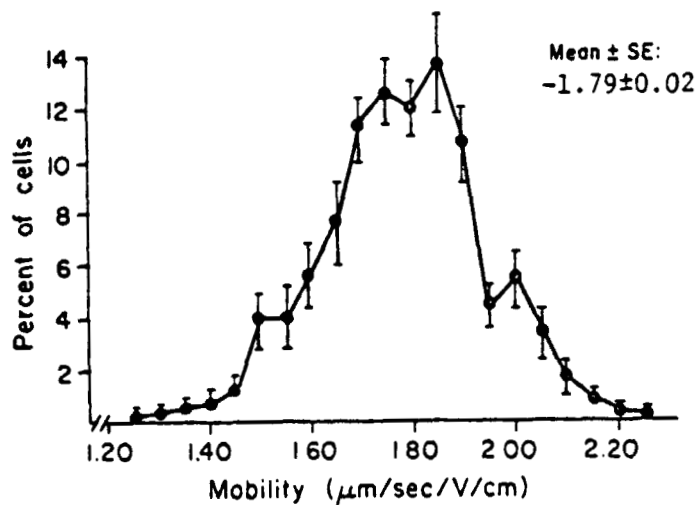


Figure 4

A TRANSPLANT PATIENT

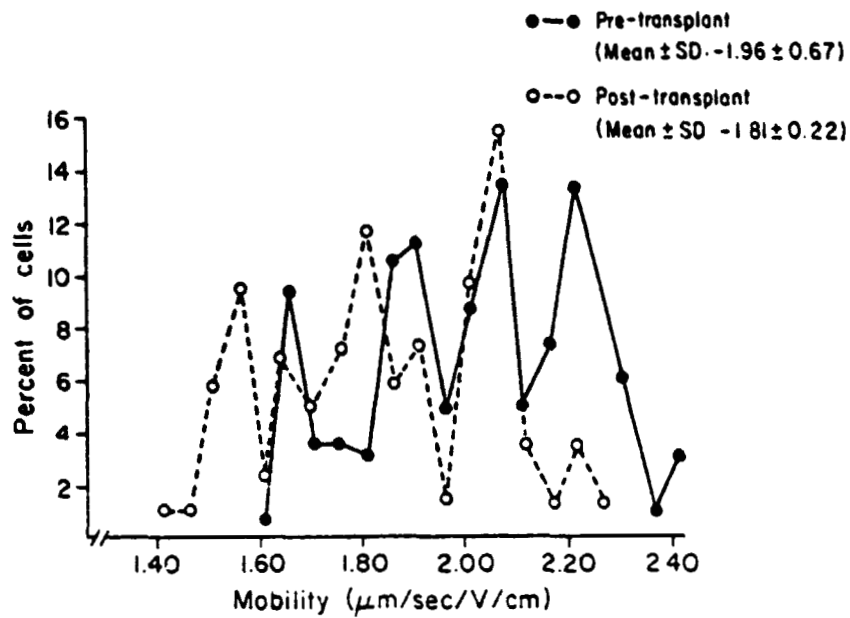


Figure 5

A TRANSPLANT PATIENT

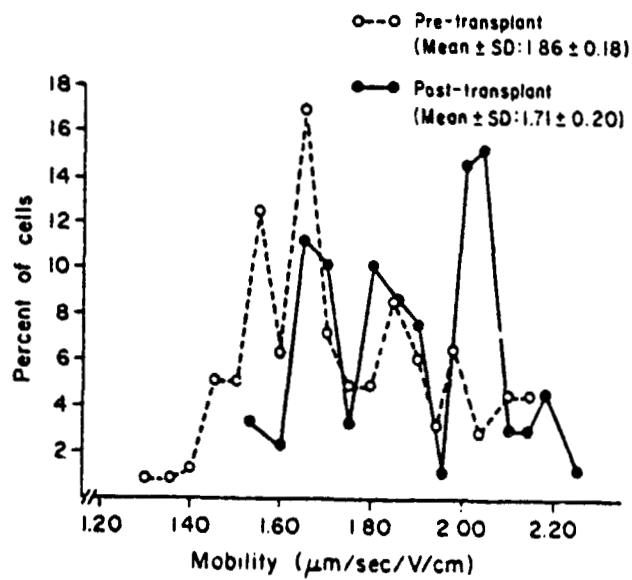


Figure 6

ELECTROPHORETIC MOBILITY OF LYMPHOCYTES IN NORMAL ADULTS

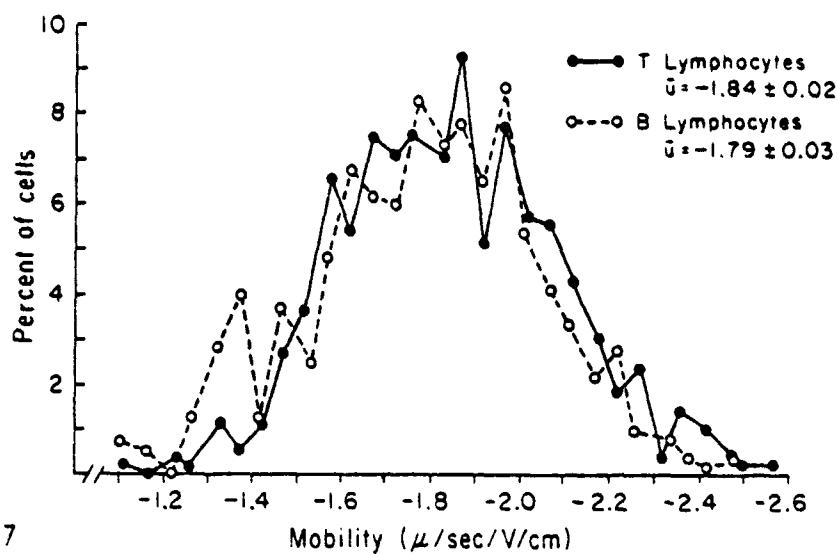


Figure 7

ELECTROPHORETIC MOBILITY OF LYMPHOCYTES IN UREMIC PATIENTS

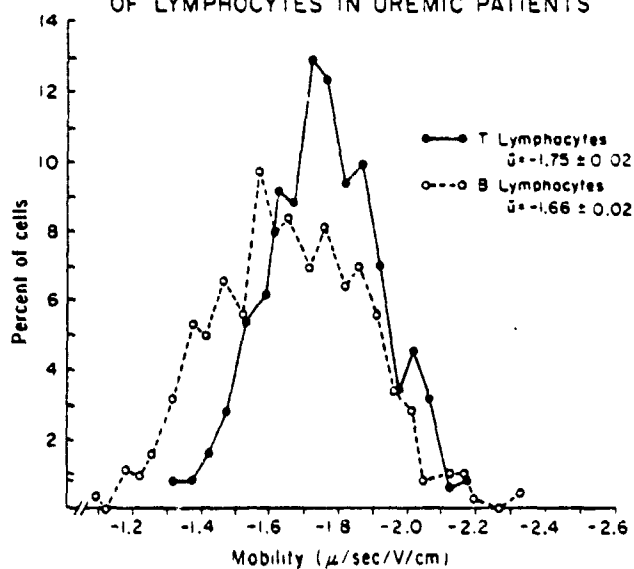


Figure 8

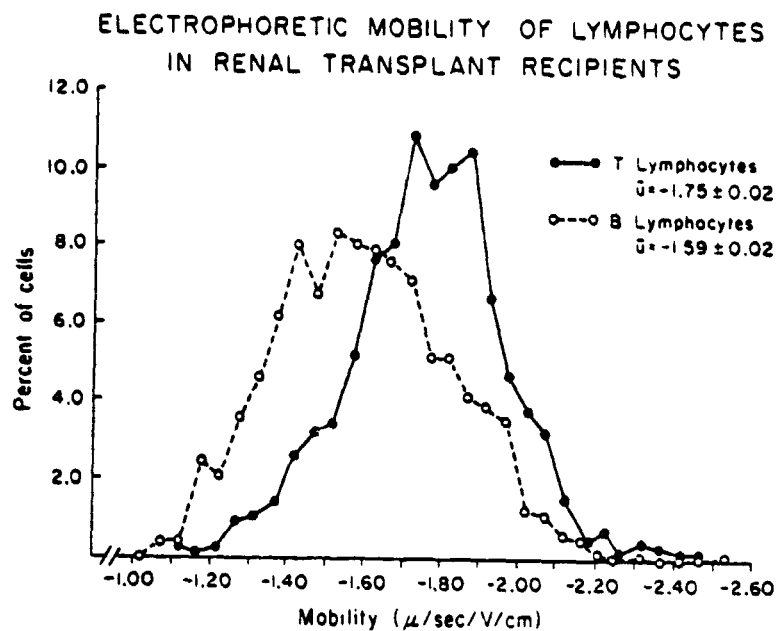


Figure 9

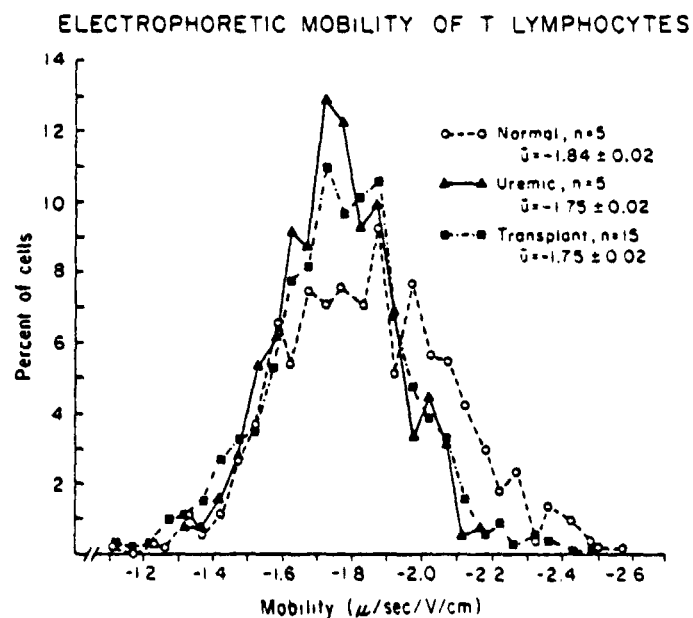


Figure 10

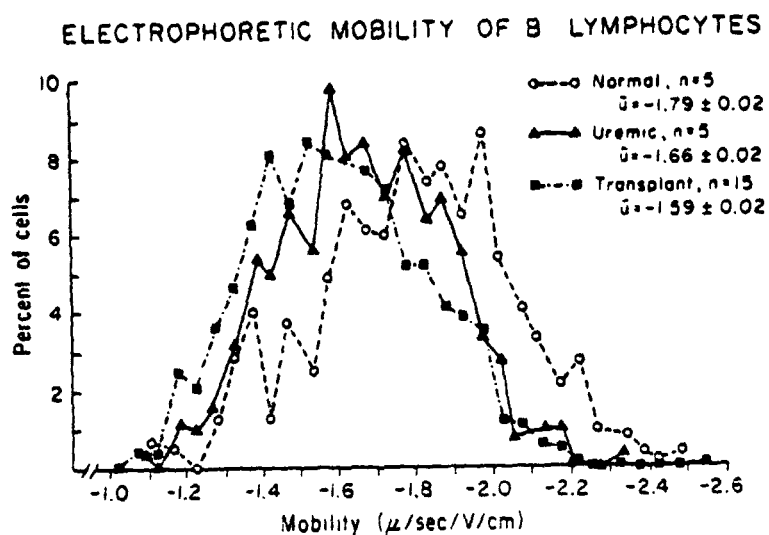


Figure 11

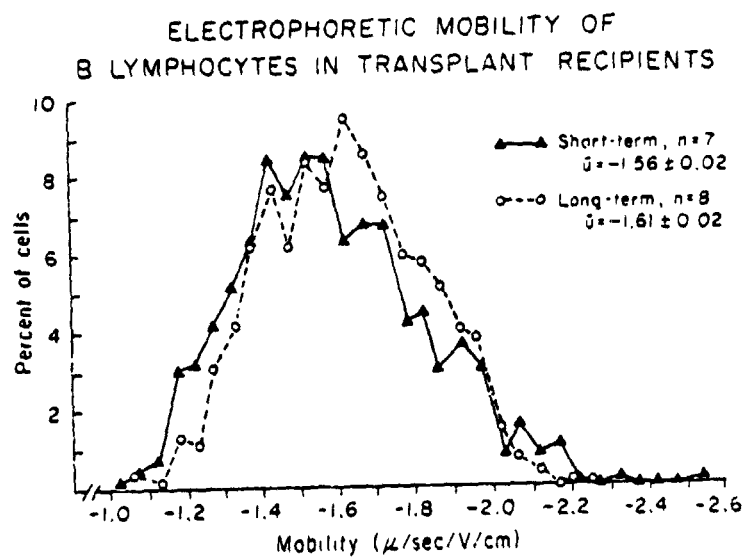


Figure 12

Table 1

REVERSIBILITY OF ELECTROPHORETIC MOBILITY OF LYMPHOCYTES

Phase I*		Phase II*	
Ionic Strength (mol. liter ⁻¹)	Mobility ($\mu\text{m/sec/V/cm}$) Mean \pm SEM	Ionic Strength (mol. liter ⁻¹)	Mobility ($\mu\text{m/sec/V/cm}$) Mean \pm SEM
0.15	1.08 \pm 0.01	0.15	1.07 \pm 0.01
0.10	1.19 \pm 0.01	0.15	1.08 \pm 0.02
0.01	2.21 \pm 0.02	0.15	1.14 \pm 0.01
0.006	2.37 \pm 0.03	0.15	1.15 \pm 0.01
0.003	2.34 \pm 0.03	0.15	1.11 \pm 0.01

* Lymphocytes are suspended in varying ionic strength media for an hour (Phase I), then resuspended in 0.15 mol. liter⁻¹ NaCl media for an hour (Phase II).

Table 2

ELECTROPHORETIC MOBILITY OF PERIPHERAL LYMPHOCYTES
FROM NORMAL SUBJECTS

Case No.	Sex	Age	EPM
			um/sec/V/cm Mean \pm SEM
1	Male	42	-1.80 \pm 0.01
			-1.74 \pm 0.01
			-1.82 \pm 0.01
2	Male	35	-1.82 \pm 0.01
3	Male	39	-1.76 \pm 0.01
4	Male	50	-1.81 \pm 0.02
			-1.82 \pm 0.02
			-1.74 \pm 0.02
5	Male	34	-1.78 \pm 0.02
			-1.72 \pm 0.02
6	Female	35	-1.77 \pm 0.01
			-1.83 \pm 0.01
7	Female	25	-1.81 \pm 0.01
Mean \pm SEM			-1.79 \pm 0.01

Table 3

ELECTROPHORETIC MOBILITY OF PERIPHERAL LYMPHOCYTES FROM
HEMODIALYSIS PATIENTS BEFORE AND AFTER
KIDNEY TRANSPLANTATION (SHORT-TERM STUDY)

Case No.	Electrophoretic Mobility ($\mu\text{m}/\text{sec}/\text{V}/\text{cm}$) (Mean \pm SEM)			Subsequent Clinical Course
	Pre-Txpl*	Post-Txpl*		
1	-1.86 \pm 0.03	-1.78 \pm 0.04	(9)**	Functioning
2	-1.81 \pm 0.02	-1.79 \pm 0.03	(6)	Functioning
3	-1.88 \pm 0.02	-1.69 \pm 0.02 -1.76 \pm 0.02 -1.88 \pm 0.02 -1.77 \pm 0.02	(7) (14) (22) (30)	Functioning
4	-1.96 \pm 0.02	-1.81 \pm 0.02	(7)	Rejected
5	-1.86 \pm 0.02	-1.71 \pm 0.02 -1.67 \pm 0.04 -1.79 \pm 0.02 -1.67 \pm 0.04	(15) (25) (31) (38)	Rejected
6	-1.80 \pm 0.02	-1.76 \pm 0.02	(7)	Rejected
Mean \pm SEM	-1.87 \pm 0.04	-1.76 \pm 0.02		

* Txpl = Transplantation

** Figures in parentheses indicate days post-transplantation

Table 4

ELECTROPHORETIC MOBILITY OF PERIPHERAL LYMPHOCYTES
FROM TRANSPLANT RECIPIENTS (LONG-TERM STUDY)

Case No.	Months Post-Txpl	Electrophoretic Mobility (u/sec/V/cm) (Mean \pm SEM)
1	31	1.79 \pm 0.01
2	13	1.69 \pm 0.02
3	27	1.62 \pm 0.02
4	13	1.61 \pm 0.01
Mean \pm SEM		-1.68 \pm 0.04

Table 5

ELECTROPHORETIC MOBILITY OF T AND "B" LYMPHOCYTES
FROM NORMAL SUBJECTS, HEMODIALYSIS PATIENTS
AND TRANSPLANT RECIPIENTS

Subjects	Electrophoretic Mobility ($\mu\text{m}/\text{sec}/\text{V}/\text{cm}$) Mean \pm SEM		Statistical Significance
	T Lymphocytes	B Lymphocytes	
Normal Subjects (N = 5)	-1.84 \pm 0.02	-1.79 \pm 0.02	NS
Hemodialysis Patients (N = 5)	-1.75 \pm 0.02	-1.66 \pm 0.02	p < 0.003
Transplant Recipients (N = 17)	-1.75 \pm 0.02	-1.59 \pm 0.02	p < 0.003

N indicates number of subjects studied.

NS indicates no statistical significance.

Arrows denote data of two groups from which statistical
evaluation was made.

Table 6

EFFECTS OF METHYLPREDNISOLONE AND TRANSPLANT
RECIPIENTS' SERA ON ELECTROPHORETIC
MOBILITY OF B LYMPHOCYTES

	Normal Serum	Normal Serum With MP*	Transplant Recipient's Serum
Case No.	Mobility ($\mu\text{m}/\text{sec}/\text{V}/\text{cm}$) Mean \pm SEM	Mobility ($\mu\text{m}/\text{sec}/\text{V}/\text{cm}$) Mean \pm SEM	Mobility ($\mu\text{m}/\text{sec}/\text{V}/\text{cm}$) Mean \pm SEM
1	-1.50 \pm 0.02	-1.69 \pm 0.02	-1.63 \pm 0.02 -1.60 \pm 0.02**
2	-1.62 \pm 0.02	-1.64 \pm 0.03	-1.69 \pm 0.02
3	-1.59 \pm 0.02	-1.61 \pm 0.02	-1.66 \pm 0.02
4	-1.50 \pm 0.02	-1.55 \pm 0.02	-1.60 \pm 0.02
5	-1.46 \pm 0.02	-1.44 \pm 0.02	-1.60 \pm 0.02
Mean \pm SEM	-1.53 \pm 0.03	-1.59 \pm 0.04	-1.63 \pm 0.02

* Succinyl methylprednisolone.

** Repeated the experiment on the same subject.

Table 7

EFFECTS OF NORMAL SERA ON
ELECTROPHORETIC MOBILITY OF B LYMPHOCYTES
FROM TRANSPLANT RECIPIENTS

Case No.	Electrophoretic Mobility ($\mu\text{m}/\text{sec}/\text{V}/\text{cm}$) Mean \pm SEM	
	Transplant Recipient's Own Serum	Normal Serum
1	-1.47 ± 0.03	-1.41 ± 0.03
2	-1.51 ± 0.02	-1.41 ± 0.02
Mean \pm SEM	-1.49 ± 0.02	-1.41 ± 0.02

Appendix I

STATISTICAL ANALYSIS:

In August 1976, raw data of electrophoretic mobility measurements on the peripheral lymphocytes from normal subjects and kidney patients were referred to Dr. Petter H. Bartels, Opital Sciences Center, University of Arizona, Tucson, Arizona. The data was sent to him to analyse the statistical significance of the observed results and specifically to answer the following questions:

1. Is the normal adult lymphocyte mobility histogram a unimodal or a polymodal distribution?
2. Do the histograms of uremic and transplant recipients differ significantly from the histograms of normal patients?
3. Are there statistically significant differences in B cells from normal, uremic, and transplant patients, and what descriptive statistics would lend themselves to such a determination?
4. What are recommended sample sizes?

Dr. Bartels provided us the following answers as the conclusion of his statistical study and recommendations:

1. The measuring precision of the technique used is about 0.02 mobility units.
2. The normal adult mobility histogram of peripheral blood lymphocytes is clearly polymodal.
3. The mobility histogram of uremic patients is clearly different from that of normal patients. The histogram is also polymodal, but exhibits a shift in the frequency of occurrence of cells

with high mobility. There does not appear to be a change in mobility of the fraction below 1.90 mobility, but a change in the number of cells in the different modes in that range. There is an increased in the number of cells in the higher mobility range. By inspection, it appears from the two profiles based on 1500 and 400 measurements respectively, that these differences are statistically significant.

4. The profiles of both B and T cell preparations each exhibit a number of peaks and shoulders, as well as minima. The most prominent features are in excellent agreement with the maxima or shoulders found in the whole peripheral blood preparations, both in normal, and in uremic patients. Somewhat surprisingly, there does not seem to be a single "B cell" peak, or a single "T" cell peak, but rather, each profile shows distinctly 4 - 7 peaks.
5. For profiles as structured as those found, and with fractions which hold but a small number of cells, a minimum number of 200 mobility measurements appears indicated, with more if possible.

Recommendations:

The analysis of electrophoretic profile data should be preceded by a preprocessing step, which may include a weighted running average option followed by an encoding of the detected peaks and minima and of shoulders. This encoding may use a chain code, or some heuristically defined grammar. It may then be followed by alignment algorithms, using a least squares fit criterion, or grammatical line-up procedures. Preprocessing will then have to be followed by

decomposition into components, either analytically, or by best fit Gaussian fitting algorithms in an iterative fashion.

Algorithms for the multivariate assessment of proportions, confidence regions and tolerance regions for the multivariate mean vectors of profiles of different patient groups exist on our system and could be adapted at rather moderate cost.

It will be necessary to design future experiments according to definite designs laid out to test specific hypotheses, to assess the run to run, patient to patient, and state of health mean squares.

Measures of similarity, and for multivariate trend description must be defined to assess post transplant profiles, relative to normal profiles, and a large data base will have to be accumulated, and set up in a well organized fashion so that eventually prognostic clues can be provided for pretransplant patients.

Appendix II

SUMMARY AND RECOMMENDATIONS

A series of experiments was conducted to evaluate the potential of lymphocyte electrophoresis as: 1) a means of monitoring the immune status of kidney transplant recipients, 2) in predicting the outcome of kidney transplants and 3) as a method for separation of lymphocyte subpopulation.

The results of this study suggest that determination of lymphocyte electrophoretic mobility does not provide either information on immune status of the host to the graft (adaptation vs. rejection) in kidney transplant recipients or means to separate two major subpopulations (T and B) of lymphocytes in normal subjects.

In certain pathologic conditions, such as patients with kidney transplants, however, lymphocyte electrophoresis does show the presence of B lymphocytes with very low electrophoretic mobility which are not present in normal subjects. Since morphological and functional characteristics as well as significance of these cells are totally unknown, separation of these lymphocytes should be attempted with a preparatory cell electrophoresis to define the immunobiologic nature of the cells.

As described in cell preparation procedure, the lymphocyte preparation referred herewith as "B" lymphocytes is, in fact not a single population of lymphocytes but it includes null cells. Since the null cells are immunobiologically a distinct lymphocyte subpopulation, electrophoretic characteristics of the null cells should be determined in both normal subjects and kidney transplant recipients. This will complete a study on the electrophoretic properties of the three known lymphocyte subpopulations.

As far as physical separation of lymphocyte subpopulations by cell electrophoretic technique is concerned, it is still possible that modification of the net surface electrical charge of lymphocytes by chemical or immunological treatment may allow such separation of multiple subpopulations of lymphocytes beyond T, B and null cells. Such treatment, particularly immunologic treatment, of cells to modify their net surface electrical charge should be explored in the future.

Since statistical analysis of the data is so complicated in this type of study, it is recommended that researchers and statisticians work together in the programming step of the experiment.

PART II STUDIES ON LYMPHOCYTE ACTIVATION

INTRODUCTION

We studied two aspects of lymphocyte activation. First, the response of human peripheral lymphocytes to a variety of mitogenic agents, that react with specific cell surface moieties, and second, we investigated in some detail the kinetics of lymphocyte commitment to DNA-replication. These studies have basic significance in understanding alterations in cell-surface topography and lymphocyte subpopulations, and should also provide insights into pathologic conditions.

A. STIMULATION OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES

Lymphocytes can be classified into different subpopulations by a variety of structural and functional parameters. Surface markers, such as immunoglobulins, Fc and C3 receptors and receptors for sheep red blood cells are most commonly used to identify subclasses of lymphocytes (1,2). Mitogens activate lymphocytes by interacting with sites on the cell surface. Although mitogens are polyclonal blastogenic agents, they are selective in their effects on subclasses of lymphocytes. For example, concanavalin A (Con A) and phytohemagglutinin (PHA), in their soluble form, stimulate only thymus-derived (T) cells; pokeweed mitogen (PWM) stimulates both bone-marrow-derived (B) cells and T cells; and bacterial lipopolysaccharide (LPS) stimulates only B cells in the mouse system (3,4). Targets for the mitogenic action of phyto-mitogens [PHA, Con A, soybean agglutinin (SBA), peanut agglutinin (PNA)] and the oxidizing agents [sodium periodate (IO_4^-) and galactose oxidase (GO)] are saccharide-containing sites on the cell surface (5-10). Some of the mitogenic agents, such as SBA, PNA and GO, exert their maximal effect only on lymphocytes that have been treated with neuraminidase (NA). This treatment exposes galactosyl residues on surface glycoproteins that then serve as targets for these mitogens (11). Thus, mitogens can be used as probes for the study of cell-surface topography.

We studied responses of human peripheral lymphocytes to a variety of mitogenic agents, including the recently discovered mitogens IO_4^- , GO, SBA and PNA. We found that responses of different individuals' lymphocytes to some mitogens (PHA, Con A, PWM) appear less variable than responses to the other mitogens (SBA, PNA, IO_4^- and GO). Moreover, the removal of adherent cells had

opposite effects on responses of purified lymphocytes to different groups of mitogens. The variability in response of different individuals' cells to some mitogens might, therefore, result from variations in adherent cell populations, rather than in the lymphocytes themselves. Extension of these studies to individuals with altered immune responses could provide insights into the pathogenesis of such alterations, and could be of value in the diagnosis and prognosis of a variety of human immunologic disorders.

MATERIALS AND METHODS

Lectins. PNA, purified from peanuts (*Arachis hypogaea*, Shulamit variety, purchased from Hazera Co., Haifa, Israel) by affinity chromatography (12) and SBA purified by affinity chromatography (13) were kindly supplied by R. Lotan and N. Sharon from the Weizmann Institute, Rehovot, Israel. Con A, twice crystallized, was purchased from Miles-Yeda Ltd., Rehovot, Israel. PHA from *Phaseolus vulgaris* (purified, HA 16) was obtained from Wellcome Research Laboratories, Beckenham, England. PWM was obtained from Grand Island Biological Company, New York.

Enzymes. NA from *Vibrio comma* was obtained from Grand Island Biological Company, New York, as a solution containing 500 units/ml (1 unit releases 1 μ g N-acetyl-neuraminic acid from acid glycoprotein at 37°C in 15 min at pH 5.5). GO was obtained from Worthington Biochemical Corp., Freehold, New Jersey. The preparation contained 30-80 units/mg---1 unit is the quantity of the enzyme that yields an absorbance of 1.0 at 420 nm, by the peroxidase chromogen (14).

Isolation of cells. Human peripheral blood lymphocytes (unfractionated cells) were obtained from healthy, normal subjects, ages 21-45, by Ficoll-Hypaque gradient centrifugation (15) and contained 70-90% lymphocytes,

10 - 30% monocytes, and a few granulocytes. Adherent cells were removed by the following procedures: (1) unfractionated cells suspended in RPMI 1640 medium containing 20% fetal calf serum (heat inactivated) were added to a 30 ml plastic syringe containing nylon wool. After incubation for 60 min at 37°C, the nonadhering cells, now enriched to 95 - 99% lymphocytes, were collected by elution with PBS. (2) Unfractionated cells (5 ml, 2×10^6 /ml) suspended in the same medium as above were incubated in plastic tissue culture petri dishes (Falcon 3003, 60 x 15 mm) for 60 min at 37°C. The non-adhering cells were then removed by repeated washings with PBS.

Fractionation of T and B Lymphocytes. T and B cells were separated by the sheep red blood cell rosetting method (16). Lymphocytes that formed rosettes and those that did not form rosettes with sheep erythrocytes are referred to as T lymphocytes and B lymphocytes respectively.

Macrophages as Adherent Cell Monolayers. Unfractionated cells (2×10^6 /ml, 0.2 ml aliquots), suspended in RPMI 1640 medium containing heat-inactivated fetal calf serum (20%), were incubated in microwells at 37°C for 60 min. The nonadherent cells were then removed by repeated washings with PBS. The macrophage monolayers were irradiated with 3,000 R. from a cesium source.

Lymphocyte Cultures. Lymphocytes (1×10^6 /ml) suspended in RPMI 1640 medium containing heat-inactivated fetal calf serum (5%) and supplemented with penicillin (100 units/ml) and streptomycin (100 µg/ml) were cultured (0.2 ml aliquots) in flat-bottom microwells (Microtest II, Falcon 3040) at 37°C in a 95% air - 5% CO₂ atmosphere for 72 hr. Twenty hr prior to the termination of the incubation, 2 µCi (in 50 µl of medium) methyl-³H-thymidine (2 Ci/m mole) were added to each culture well, and its incorporation into DNA was measured (17).

The results (cpm) are expressed as the mean of the duplicate cultures.

MITOGENIC STIMULATION OF CELLS

Lectins. Cells were incubated with the lectins at the following final concentrations (per ml): PHA, 2 μ g; Con A, 2 μ g; PWM, 10 μ l of reconstituted solution; SBA, 50 μ g; PNA, 125 μ g.

Periodate. Cells ($10 - 20 \times 10^6$ /ml), suspended in PBS, were incubated with NaIO_4 (1 mM) at 0°C for 30 min, followed by washing with PBS to remove excess reagent.

Enzymes. Cells ($10 - 20 \times 10^6$ /ml) suspended in PBS were treated with NA (50 units/ml) and GO (1.4 units/ml) at 37°C for 30 min with shaking, followed by two washings with PBS to remove excess reagent. In different experiments, cells were treated with either enzyme alone or with both enzymes simultaneously.

RESULTS

Responses of Unfractionated Lymphocytes to Different Groups of Mitogens. Responses of normal human peripheral blood lymphocytes to the following three distinct groups of mitogens were determined: Group I: PHA, Con A and PWM (lectins whose binding sites are fully exposed on the cell surface); Group II: SBA and PNA (lectins that bind to galactosyl moieties of cell-surface glycoproteins and exert their maximal effect only after removal of sialic acid residues); and Group III: IO_4^- and GO (oxidizing agents that introduce aldehyde groups on the cell surface by oxidation of sialyl and galactosyl residues respectively).

SBA and PNA are mitogenic for human lymphocytes after treatment with NA (Table I). SBA had a mitogenic effect on most individuals' lymphocytes even

without NA treatment. However, the stimulating effect of SBA was more pronounced on NA-treated cells. PNA, on the other hand, failed to stimulate lymphocytes that had not been treated with NA, as was also reported recently (10).

GO, like the galactosyl-binding lectins, had its greatest effect only after cells were treated with NA. These findings confirm and extend previous reports (18,19). The degree of enhancement of ³H-thymidine incorporation of NA-treated cells was, however, greater for SBA than for GO; that is, NA+SBA: SBA > NA+GO: GO (Table I).

Responses of lymphocytes to Group II and Group III mitogens were highly variable (Table I), both among different individuals and in the same individual at different times. While some variability was also seen in responses to Group I mitogens (Table I), it was generally less pronounced (see SD for various groups). This variation in response to Group III mitogens has been previously noted (17-21).

Even though SBA, PNA and GO exert their mitogenic effect by interacting with galactosyl residues on cell-surface glycoproteins, there was no obvious positive correlation among responses to these mitogens. Indeed, in some instances, there was an inverse relationship between, for instance, NA+SBA and NA+GO (see, for example, PK-2, ALR, JC-1 and RRR [Table I]).

Responses of T and B Lymphocytes to the Different Groups of Mitogens. Group I mitogens, as demonstrated by many others (3), stimulate only T cells (Fig. 1). Group II mitogens, SBA and PNA, are selective T cell mitogens for mouse lymphocytes (22), and our findings (Fig. 1) indicate that they are also selective T cell mitogens in the human system. An interaction of SBA with human T lymphocytes has been recently reported (23).

Responses of both separated T and B lymphocytes to Group III mitogens were markedly reduced. Since previous reports have indicated that this group of mitogens is exquisitely sensitive to removal of adherent cells (18,21,24), we determined the effect of removal and replacement of adherent cells on responses of T and B cells to IO_4^- and GO. Results summarized in Figures 2 and 3 clearly indicate that these mitogens are also selective for T cells, as was previously reported for mouse lymphocytes (22,25). Previous studies have shown that untreated lymphocytes are stimulated in the presence of aldehyde-modified macrophages, as well as when the lymphocytes themselves are aldehyde-modified and incubated in the presence of untreated macrophages (18,21,24). Figures 2 and 3 indicate that in either of these distinct experimental conditions, only T cells proliferate. The slight response of the B cell population to NA+GO might result from contamination of the preparation with T cells.

Responses of T lymphocytes to Group I mitogens (PHA, Con A and PWM) were slightly enhanced when compared with responses of unfractionated lymphocytes to these agents. This degree of enhancement could be accounted for by enrichment of the cell suspension with T cells. Responses of separated T cells to Group II mitogens (SBA and PNA), on the other hand, were significantly higher than responses of comparably stimulated unfractionated cells. This increase could not be explained by simple enrichment of the cell suspensions with T cells. As noted above, responses of T cells in the absence of adherent cells to Group III mitogens (IO_4^- and GO), in contrast to the other mitogens, were consistently and significantly reduced (Figure 1).

Enhancement of T cell responses to Group II mitogens might be attributed to a depletion of adherent cells. The method used for T cell separation re-

moves macrophages as well as B cells (16). We therefore determined the effect of simply removing adherent cells from unfractionated lymphocytes on their blastogenic responses to these various mitogens.

Effect of Adherent Cells on Lymphocyte Responses to the Different Groups of Mitogens. Figure 4 shows the effects of depleting adherent cells on lymphocyte responses to the three groups of mitogens. This procedure clearly resulted in a change in the response of the remaining, nonadherent cells to the various mitogens. These changes were in the same direction as noted above (Fig. 1) for the alterations in the purified T cell responses. Thus, merely removing adherent cells resulted in opposite effects on responsiveness to Group II and III mitogens. Stimulation was markedly enhanced in response to Group II mitogens, was variably decreased to NA+GO, but consistently decreased to IO_4^- (Group III mitogens). In some instances, depletion of adherent cells markedly diminished IO_4^- response while NA+GO stimulation was not decreased. Stimulation by Group I mitogens was only slightly affected by depletion of adherent cells. Similar results were obtained by removing adherent cells by either adhesion to nylon wool or to petri dishes.

These alterations in response of lymphocytes after removal of adherent cells were also measured by determining 3H -uridine and ^{14}C -leucine incorporation. Results were similar to those obtained for 3H -thymidine incorporation (Table II).

DISCUSSION

Sites located on cell membranes are the primary targets for mitogens. Since different lymphocyte subpopulations might vary in their surface membrane

properties, various mitogens might affect them differently. This could be a valuable characteristic in identification and characterization of such groups of lymphocytes. In this study, we investigated the blastogenic effects of a variety of mitogenic agents on normal human peripheral lymphocytes. The mitogens tested may be classified into three groups: I. The phytomitogens that exert their effect by binding to fully exposed cell-surface sites (PHA, Con A, PWM); II. The phytomitogens that bind to galactosyl residues and exert their maximal effect on NA-treated cells; and III. Oxidizing agents, IO_4^- and GO, that induce mitogenesis by generation of aldehyde moieties on either sialyl or galactosyl residues respectively. Mitogenic properties of the agents in Group I have been extensively investigated before and are included in this study for comparison to the other mitogens.

As shown in Table I, SBA and PNA exert their maximal effect only after lymphocytes have been treated with NA. SBA was also found to stimulate untreated lymphocytes, but to a much lesser degree. PNA, on the other hand, failed to stimulate lymphocytes that had not been treated with NA. This finding suggests the presence of sites on intact (i.e., cells not treated with NA) human peripheral lymphocytes that are recognized by SBA but not by PNA. We could not, however, exclude the possibility that the failure of PNA to stimulate intact lymphocytes results from steric hindrance of this lectin reaching its binding site. The presence of distinct sites is suggested by the recent isolation of a "T antigen" from NA-treated porcine lymphocytes that specifically binds PNA (26).

As was noted before, there are marked species differences in lymphocytes to the blastogenic response of SBA and PNA. PNA fails to stimulate murine

lymphocytes even after their treatment with NA (10). On the other hand, PNA is mitogenic to NA-treated human peripheral lymphocytes and has no effect on intact cells. SBA is mitogenic to intact lymphocytes from humans but not from mice. Lymphocytes from both species are rendered highly responsive to the lectin after their treatment with NA. These findings illustrate the differences in the membrane sites present on lymphocytes from different species.

IO_4^- and GO have been shown to be mitogenic for lymphocytes isolated from a variety of mammalian species. GO failed, however, to stimulate intact murine lymphocytes and was mitogenic only after cells were treated with NA. Our findings confirm and extend the recent observations that GO exerts a mitogenic effect on human lymphocytes even without NA treatment (18,19).

Table I indicates that there is an extensive variability in the response of human lymphocytes to SBA and PNA both among individuals and in the same individual tested at different times. A similar variability has previously been found in the response to GO and IO_4^- (18,21), and again documented here. Variability in response to PHA and Con A was, in contrast, less pronounced. Since crude lymphocyte preparations contain multiple types of cells that could affect blastogenesis by various cell-to-cell interactions, we studied the blastogenic response of defined cell types to the different mitogens. The responses of purified B and T lymphocytes to three groups of mitogens is shown in Figure 1. The results clearly demonstrate that both PNA and SBA are T cell mitogens. Blastogenic responses to IO_4^- and GO, however, of both B and T cells were markedly diminished. Since a strict requirement for macrophages in the IO_4^- and GO systems has been clearly documented and since the method we employed for T and B cell purification depletes the T cell fraction of macrophages,

investigated the effect of adherent cells on the response of B and T cells to these mitogens. Results summarized in Figures 2 and 3 demonstrate that GO and IO_4^- are T cell mitogens. T cells were the responding cells when either the macrophages or the T cells themselves were modified by the oxidizing agent.

A surprising finding was that the blastogenic response of purified T cells to SBA and PNA was markedly enhanced to an extent that could not be accounted for by enrichment of cell suspensions with T cells. T cell responses to PHA and Con A, when compared to responses of unfractionated cells, were only moderately affected. Similar increments in SBA and PNA response were found after passing unfractionated cells through nylon-wool columns and after allowing cells to adhere to petri dishes. These procedures deplete cell suspensions of macrophages as well as B cells. The depletion of either or both of these cell types could be responsible for the observed enhanced response to SBA and PNA. Preliminary reconstitution experiments (by adding macrophages or B cells to T cell preparations) have so far not resulted in suppressing SBA and PNA responses.

The enhanced lymphocyte response to SBA and PNA after removal of adherent cells was also documented by measuring 3H -uridine and ^{14}C -leucine in addition to 3H -thymidine incorporation. All three precursors gave similar results (Table II). This rules out the possibility, recently documented in the mouse system (27), that thymidine might be released into the medium from macrophages and dilute the 3H -thymidine used to assess cell proliferation.

In conclusion, the response of T cells to different groups of mitogens is either enhanced, depressed, or unchanged by the presence of other cell

types. It is possible that these different responses might account for variations in lymphocyte reactivity to a variety of stimuli in normal as well as in pathologic states.

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Legends to Figures

Figure 1. Responses of peripheral blood lymphocytes (PBL), T-cells and B-cells to Con A, PHA, NA+Con A, NA+SBA, NA+PNA, GO, NA+GO and IO_4^- . Each connected series of points represents response of a different individual's cells.

Figure 2. Lymphocyte and macrophage (MØ) requirements for IO_4^- stimulation. PBL- = PBL untreated; PBL+ = PBL treated IO_4^- ; T-, B- = T or B cells, untreated; T+, B+ = T or B cells treated with IO_4^- ; MØ- = macrophages untreated; MØ+ = macrophages treated with IO_4^- .

Figure 3. Lymphocyte and macrophage (MØ) requirements for NA+GO stimulation. Experimental conditions were similar to those outlined in Figure 2, except that the cells were treated with NA+GO.

Figure 4. Responses of peripheral blood lymphocytes (PBL) and PBL with adherent cells, or MØ, removed (PBL-MØ) to Con A, NA+Con A, PHA, NA+PHA, NA+SBA, NA+PNA, GO, NA+GO and IO_4^- . Open points = adherent cells removed by adhesion to petri dishes; closed points = adherent cells removed by passage through a nylon wool column.

Tables

Table I. CPM ^3H -Thymidine Incorporation/Culture

Table II. ^3H -Thymidine (Td), ^3H -Uridine (Ud) and ^{14}C -Leucine (LEU)
Incorporation/Culture before and after removal of
adherent cells

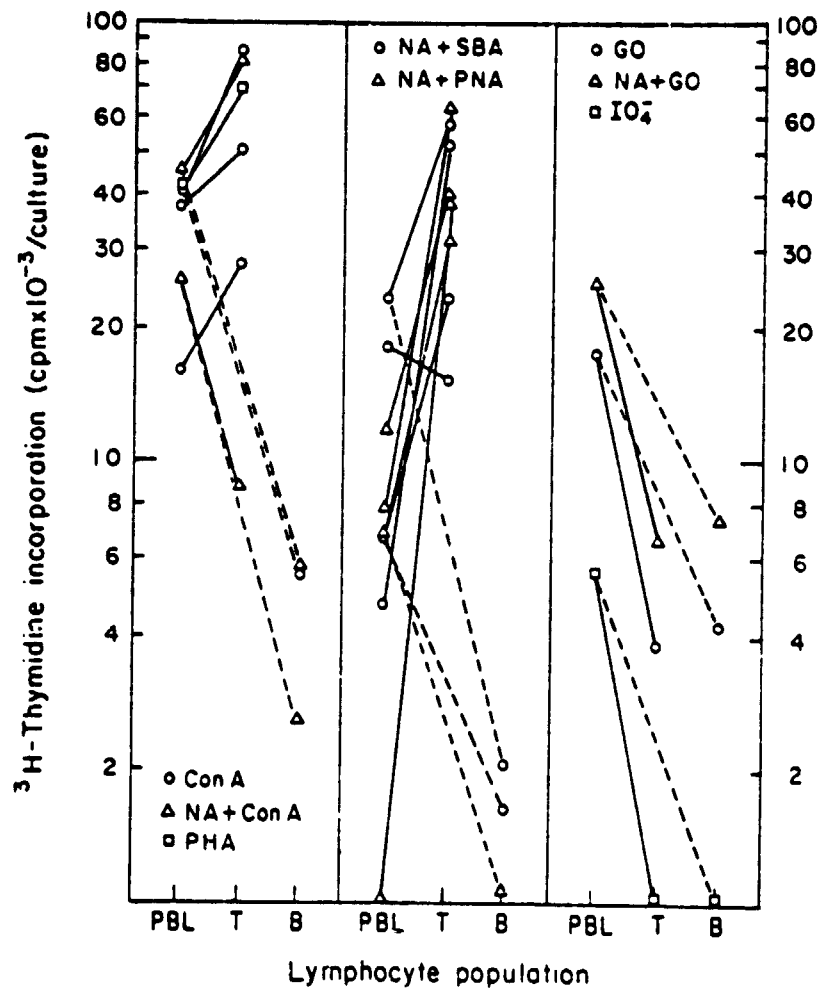


Figure 1

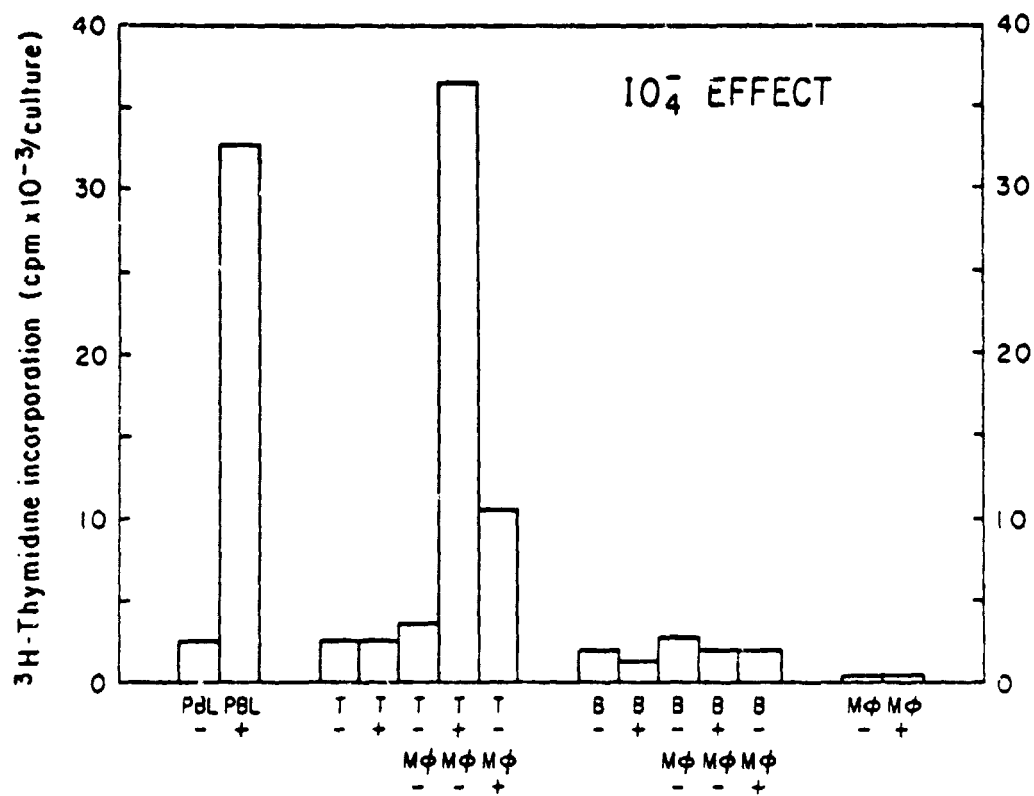


Figure 2

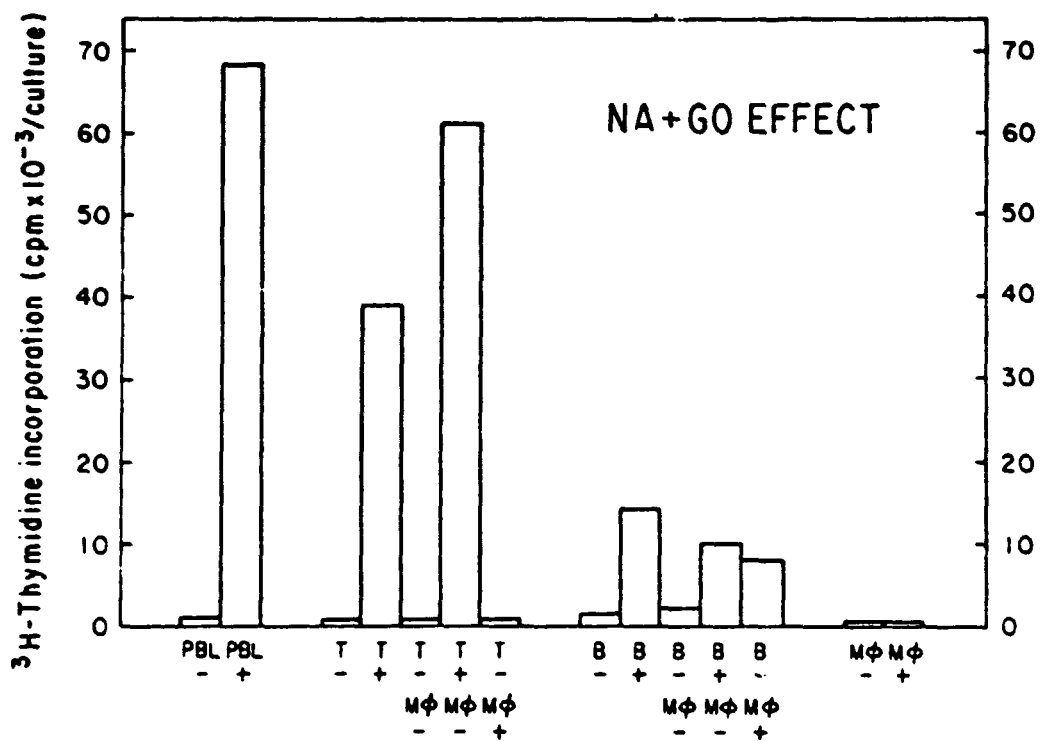


Figure 3

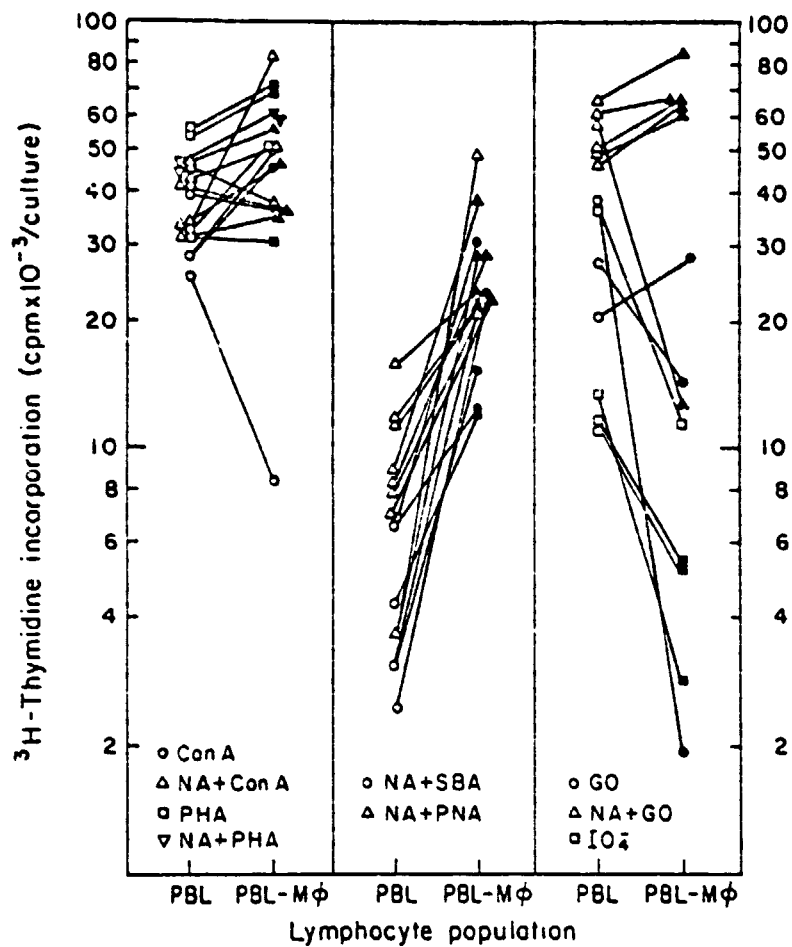


Figure 4

TABLE I

³H-THYMIDINE INCORPORATION/CULTURE

Donor*	Untreated	ConA	PHA	PWM	NA	SBA	PNA	NA SBA	NA PNA	GO	NA+GO	IO ₄
1 PK-1	386	26103	89151	14434	826	9421	397	---	---	34660	110204	24600
2 PK-2	576	16244	83897	18761	956	2651	642	44076	36171	6635	9189	34051
3 ALR	631	44989	51437	37974	453	787	614	5468	9827	7044	83429	7009
4 JC-1	2405	39568	62839	28049	453	7739	2074	11618	4205	41119	62749	16108
5 JC-2	4810	66967	133126	36152	4298	21712	5743	77140	40173	2334	66676	50080
6 RRR	1345	67941	73648	72906	1478	3422	1837	6949	33374	40077	56565	10017
7 TM-1	570	30925	48960	21571	668	1080	506	1862	2022	24483	25133	13745
8 TM-2	578	13838	54533	22387	722	503	406	15661	13165	582	7545	13437
9 KHS-1	789	22592	39596	16387	625	1866	527	12272	11038	20262	28501	16290
10 KHS-2	479	24284	60718	16029	599	1223	527	17219	27884	37076	50517	25920
11 LT-1	1812	37157	53380	25131	1457	2601	1076	16042	17038	34335	42484	44216
12 LT-2	1087	37314	60105	24769	479	8059	485	23513	4554	4871	5713	35780
13 RK-1	759	39776	66326	19658	227	5576	404	30391	10297	---	---	33574
14 RK-2	1564	60642	84469	14080	2502	26566	2513	113726	35562	2053	8447	77317
15 RS-1	2956	111112	186482	76854	4218	17782	2844	65734	66386	30635	82965	43344
16 RS-2	1137	33214	101691	28168	920	9933	1119	38978	15798	14496	17863	49587
17 AN	3135	30463	65094	14541	3477	9006	2919	43713	38724	23897	42528	31078
18 DM	573	55593	88671	22438	530	1950	357	2220	8395	9480	36233	4404
19 DW	1337	52008	59260	27832	903	7634	980	5662	27778	29756	20021	5496
Mean	1417	42674	77020	28322	1357	7343	1367	29572	22355	20211	42598	28214
±SD	1165	22969	34454	17767	1288	7409	1372	30214	16998	14413	30705	19086

* 1 and 2 after donors' initials indicates the same donor tested on different days.

TABLE II

³H-Thymidine (Td), ³H-Uridine (Ud) and ¹⁴C-Leucine (LEU)
Incorporation/Culture before and after removal of
adherent cells^a

<u>Donor</u>	<u>Isotope</u>		<u>NA</u>	<u>NA + GonA</u>	<u>NA + SBA</u>	<u>NA + PNA</u>	<u>NA + GO</u>
1	³ H-Td	Pre ^b	651	41740	2385	8951	50602
		Post	1195	35218	22443	33934	63183
	³ H-Ud	Pre	3665	41618	6715	7623	44134
		Post	5583	31914	13791	24151	48774
	¹⁴ C-LEU	Pre	1023	4774	1625	1573	12562
		Post	817	6653	3288	3752	16443
			<u>Untreated</u>	<u>PHA</u>	<u>IO₄</u>		
2	³ H-Td	Pre	642	56,485	11,574		62909
		Post	1559	71,410	5,429		67125
	³ H-Ud	Pre	2027	---	14,029		42230
		Post	3731	---	6,900		38761
3	³ H-Td	Pre	369	---	13,403		65860
		Post	736	---	2,837		86204
	³ H-Ud	Pre	1599	---	12,526		49276
		Post	228	---	3,946		46584

a. Adherent cells were removed by passage through a nylon wool column.

b. Pre and Post = Before and After removal of adherent cells.

B. KINETICS OF LYMPHOCYTE COMMITMENT TO DNA REPLICATION INDUCED BY CONCAVALIN A AND WHEAT GERM AGGLUTININ AT SUB- AND SUPRA-OPTIMAL CONCENTRATIONS

Mitogens induce lymphocyte proliferation by interacting with, or modifying, saccharide-containing sites on the cell membrane (1-2). The proliferation induced by mitogenic agents, especially the phytomitogens, exhibits a characteristic dose-response curve. Extent of blastogenesis is proportional to the amount of lectin added up to a maxima, but further increases in lectin concentration result in a diminishing stimulatory effect. This inhibition at supra-optimal lectin concentrations cannot be ascribed to cell damage, since removal of excess mitogen re-establishes responsiveness (3). It has recently been demonstrated that concanavalin A (Con A) at high concentrations restricts lateral mobility of various cell-surface structures (4). A variety of biochemical alterations occurring soon after stimulating lymphocytes with mitogens exhibit a dose-response curve similar to that of mitogen-induced lymphocyte transformation (5-6). Thus, it has generally been considered that these supra-optimal lectin concentrations do not initiate a triggering signal.

Previous studies have also indicated that lymphocyte transformation induced by lectin occurs in two phases. During the first 24 hours (approximately) after lectin addition, blastogenesis is mitogen-dependent; whereas after this time, cells become committed to DNA replication and the progression of blastogenesis is mitogen-independent (7-10). A recent report demonstrates that late addition of α -methyl-D-mannoside (α MM) to lymphocyte cultures treated with supra-optimal concentrations of Con A results in a marked stimulatory response (11). It was concluded that a positive signal is initiated by these high concentrations of Con A, but that they fail to induce DNA replication because of a simultaneous inhibitory signal. The potential significance of

these findings in understanding the nature of the triggering signal for lymphocyte activation prompted us to investigate this system in some detail.

We examined mitogenic properties of Con A as well as those of wheat germ agglutinin (WGA) in terms of the kinetics of lymphocyte commitment to DNA replication. Con A is a well-characterized phytomitogen, and WGA has recently been found to be mitogenic for human peripheral lymphocytes (12). Our data show that the apparent commitment of phytomitogen-treated cells to DNA replication at 24 hours is dependent on mitogen concentration. At this time, addition of competing saccharides almost completely eliminates blastogenesis of cells treated with sub-optimal concentrations of mitogen and, as was reported recently (11), enhances the response of cell treated with supra-optimal concentrations of mitogens. Addition of α MM to cells after only 4 hr treatment with supra-optimal concentrations of Con A results in maximal blastogenesis. The data indicate that cell-bound Con A is rendered inaccessible to the competing saccharide by a time and temperature dependent process. Initiation of blastogenesis by adding competing saccharide to cells treated with supra-optimal concentrations of Con A appears to result from a reduction in cell-bound Con A to an effective stimulatory concentration. Commitment of lymphocytes to DNA replication by phytomitogens may require longer cell contact than has heretofore been recognized.

MATERIALS AND METHODS

Lectins. WGA, purified by affinity chromatography (13) was kindly supplied by R. Lotan and N. Sharon from the Weizmann Institute of Science, Rehovot, Israel, and was also purchased from Pharmacia Fine Chemicals and from

Calbiochem. Con A, twice crystallized, was purchased from Miles-Yeda Ltd., Rehovot, Israel. α MM, N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) were purchased from Pfanstiehl Laboratories, Inc., Waukegan, Illinois.

Isolation of Cells. Human peripheral blood lymphocytes (unfractionated cells) were obtained from healthy, normal subjects, age 21-45, by Ficoll-Hypaque gradient centrifugation (14) and contained 70-90% lymphocytes and 10-25% monocytes.

Lymphocyte Cultures. Lymphocytes (1×10^6 /ml) suspended in RPMI 1640 medium containing heat-inactivated fetal calf serum (5%) and supplemented with penicillin (100 units/ml) and streptomycin (100 μ g/ml) were cultured (0.2 ml aliquots) in flat-bottom microwells (Microtest II, Falcon 3040) at 37°C in a 95% air / 5% CO₂ atmosphere for 72 hr. Twenty hr prior to the termination of the incubation, 2 μ Ci (in 50 μ l of medium) methyl-³H-thymidine (2 Ci/m mole) were added to each culture well and incorporation into DNA was measured (15). Results (cpm) are expressed as the mean of duplicate cultures.

α MM, GlcNAc, and GalNAc were added to cell cultures as a 1M solution to a final concentration of 0.1M, 0.05M, and 0.05M respectively.

RESULTS

Mitogenic Activity of WGA. The effect of WGA on ³H-thymidine incorporation in peripheral blood lymphocytes was examined in cells from 11 normal individuals. Three preparations of WGA (see Materials and Methods) were

tested, and all were mitogenic. The experiments reported here utilized the WGA-Pharmacia preparation. ^3H -Thymidine incorporation was less than that seen with Con A stimulation (see below), but was nevertheless consistent and reproducible. The effect of addition of the competing monosaccharide, GlcNAc, 24 hr after adding WGA on ^3H -thymidine incorporation was dependent upon the WGA concentration (Figure 1). At high or supra-optimal WGA concentrations (50 $\mu\text{g/ml}$), ^3H -thymidine incorporation was enhanced by GlcNAc, while at low concentrations (10 $\mu\text{g/ml}$) GlcNAc resulted in decreased ^3H -thymidine incorporation (Figure 2 C,E,F). At intermediate WGA concentrations (10 - 20 $\mu\text{g/ml}$), concentrations that resulted in optimal stimulation, there was little or no effect of addition of GlcNAc (Figure 1-- 10 $\mu\text{g/ml}$ in A and B; 20 $\mu\text{g/ml}$ in D-F).

WGA stimulation was also measured after 120 hr of incubation and was markedly enhanced over that at 52 hr (Figure 2), in accord with a recent report showing a delayed maximum activity of WGA stimulation (12). In this experiment, the differential effect of GlcNAc addition at 24 hr is clearly evident: depression of ^3H -thymidine incorporation at 10 $\mu\text{g/ml}$, little difference at 20 $\mu\text{g/ml}$, and enhanced incorporation at 50 $\mu\text{g/ml}$.

Specificity of Saccharide in the Induction of Enhanced Mitogenic Response to WGA at High Concentrations. The specificity of GlcNAc in inducing an enhanced mitogenic response to WGA at high WGA concentrations (50 $\mu\text{g/ml}$) was examined. This saccharide specifically inhibits WGA binding to cell-surface glycoproteins (1) and WGA-induced blastogenesis (12). Twenty-four hr after stimulation of cultures with WGA, αMM , GalNAc, or GlcNAc were added.

The cultures were pulsed with ^3H -thymidine at 120 hr to ensure maximum amplitude of the response. The results showed that thymidine incorporation in GlcNAc-treated cultures was 140,000 cpm, whereas it was less than 2,000 cpm in cultures treated with the other monosaccharides, αMM and GalNAc.

Effect of Preincubation of Lymphocytes on Their Blastogenic Response to WGA.

Since the commitment to DNA replication induced by WGA appears to be dose-dependent (Figure 1), we considered the possibility that addition of the competing saccharide reduces the effective WGA concentration in the system to an optimal mitogenic level in the case of high concentrations (50 $\mu\text{g/ml}$), and to a sub-mitogenic level in the case of low concentrations (10 $\mu\text{g/ml}$). This in turn would suggest that cells treated with high-dose WGA are not committed to DNA replication at 24 hr, but are merely being "incubated" for that time, with no effective mitogenic stimulus. Since preincubation of lymphocytes in the absence of lectin has been shown to enhance their blastogenic response to lectins (16), we examined the effect of preincubation of cells on their response to WGA with and without the addition of GlcNAc at 24 hr. Figure 3 indicates that preincubation at 37°C markedly enhances the response to WGA, especially when GlcNAc is added at 24 hr. Thus, at least two effects may be promoting enhanced DNA replication at high lectin concentrations when the competing saccharide is added to the cultures: [1] reduction of lectin concentration to a mitogenic level; and [2] the amplification of the response by preincubation.

Effects of Competing Saccharide Addition and Preincubation on Lymphocyte Activation Induced by Con A. The kinetics of cell commitment to DNA replication were further investigated using Con A, a well-characterized T-cell-specific

phyto mitogen. Cultures were treated with varying concentrations of Con A, and α MM was added to replicate cultures. Cultures were pulsed with ^3H -thymidine at 52 hr and harvested at 72 hr. Figure 4 indicates the results of 3 experiments in 3 individuals. The differential effects of competing saccharide addition at low and high lectin concentrations are clearly evident. Note the differences in ^3H -thymidine incorporation at 2 $\mu\text{g}/\text{ml}$ and at 20 $\mu\text{g}/\text{ml}$ in cultures with and without α MM. Addition of α MM to cultures treated with low concentrations of Con A results in a depressed response, addition to those treated with optimal levels results in no change, and addition to those treated with high or supra-optimal levels of Con A results in an enhanced response.

Preincubation of cells amplifies the response to Con A (Figure 4), as has previously been shown (16). The effects of both preincubation and α MM addition are depicted in this figure. Preincubation increases responsiveness to low concentrations of lectin, and apparently shifts the dose-response curve far to the left. The peak response with addition of α MM is greater in the pre-incubated cells, and the response, even with α MM addition, is diminishing at the high Con A concentrations.

An experiment to determine the kinetics of the preincubation effect is shown in Figure 5. 1 μg of Con A was added either directly or to cells pre-incubated for 24 hr, and ^3H -thymidine incorporation measured at 1, 2, 3, and 4 days. The response of preincubated cells is greater at each time period after 1 day. Incorporation of ^3H -thymidine in preincubated cells appears to be approximately the same as the value obtained 24 hr later in the cells tested directly.

Effects of α MM addition to Con A-treated cultures at times earlier than 24 hr are shown in Figure 6. When α MM was added either prior to Con A treatment

or after 1 hr incubations of cells at 0° C in the presence of Con A, the dose-response curve is identical. Even under these conditions, high concentrations of Con A initiate a small but significant response. The response is greater when cultures are incubated at 37° C for 1 hr prior to α MM addition. After 4 hr exposure to Con A at supra-optimal concentrations, addition of α MM results in essentially the same effect as addition at 24 hr. Thus, addition of α MM at both time intervals resulted in maximal stimulation, to the same extent as that induced by optimal concentrations of Con A in the absence of α MM.

DISCUSSION

Data presented in Figures 1, 2, and 4 indicate that the time at which initiation of DNA synthesis is no longer suppressed by addition of competing saccharides depends on the lectin concentration for both WGA and Con A. Even after 24 hr exposure to lectins, DNA replication in cells treated with sub-optimal mitogen concentrations is virtually eliminated by addition of the competing saccharide. An apparent commitment to DNA replication at 24 hr is found only with optimal lectin concentrations. Removal of high, supra-optimal levels of either WGA or Con A at 24 hr by addition of competing saccharide results in an enhanced response, as was previously reported for Con A (11) and corroborated, and extended to WGA, in our experiments. Thus, removal of lectin at 24 hr markedly and differentially, depending on the lectin dose, alters blastogenic responses. These alterations could result from reduction of lectin concentrations from inhibitory to mitogenic levels when supra-optimal concentrations are used, and from marginally mitogenic to nonmitogenic levels when sub-optimal lectin concentrations are used.

Our data strongly suggest that residual mitogen remaining on the cells after treatment with competing saccharide is responsible for the blastogenic response of cells that have been blocked by high concentrations of lectin. When α MM is added 4 hr after cells are treated with Con A over a wide dose range, response to optimal Con A concentrations is markedly inhibited, in accord with previous studies on lymphocyte commitment (7-10). However, cells treated with supra-optimal Con A concentrations behave as though they were committed. Either commitment is imposed on cells by high concentrations of Con A, but not by optimal concentrations, or α MM only partially removes lectin, reducing supra-optimal concentrations to mitogenic ones that remain in contact with the cell. The latter possibility appears more likely. When α MM is added prior to Con A, or when cells are incubated at 0° C with Con A for 1 hr prior to α MM, the dose-response curve reveals a minimal but significant response at high lectin concentrations (Figure 6). When α MM is added to cells incubated with Con A at 37° C for 1 hr and 4 hr, the intensity of the response progressively increases. The Con A binding sites would appear to be rendered inaccessible to α MM by a time and temperature dependent process. Thus, removal of lectin by competitive inhibition with the monosaccharide that specifically competes with its binding to cell-surface glycoproteins may not accurately determine kinetics of cell commitment to DNA replication. Our data would indicate that the first, or mitogen-dependent, phase of lymphocyte stimulation may be much longer than the approximate 24 hr previously reported.

Preincubation of lymphocytes in the absence of mitogen markedly potentiates their response to subsequent mitogenic stimulation (Figures 2 and 5). This potentiation is reflected in part by an increase in blastogenesis by lectin

at sub-optimal concentrations, as was previously reported (16). More striking, however, is the finding that preincubated cells are apparently committed to DNA replication after 24 hr contact with sub-optimal concentrations of WGA or Con A. Addition of competing saccharide to cells treated with low Con A concentrations (2 μ g/ml) has no effect if the cells are preincubated, but results in marked inhibition in the absence of preincubation (Figure 4). The observed stimulation of lymphocytes by adding the competing saccharide at 24 hr to cultures treated with supra-optimal lectin concentrations could also be potentiated by preincubation of cells for 24 hr prior to exposing them to effective or mitogenic concentrations of Con A.

SUMMARY

Cell commitment to DNA replication as measured by the addition of competing saccharide to lectin-treated cells is dependent on mitogen concentration. An apparent cell commitment is obtained at 24 hr with optimal lectin concentrations, but commitment is not seen with sub-optimal concentrations. Cells are committed by lower concentrations of lectin if they are preincubated at 37°C in the absence of mitogens. The marked response of the cell to supra-optimal concentrations of lectin when α MM is added at 24 hr could be explained by the following: [1] partial removal of nonmitogenic doses of lectins to stimulatory levels; and [2] the potentiation effect of preincubation in the absence of an effective mitogenic stimulus. Addition of α MM after only 4 hr to cells treated with supra-optimal concentration of Con A results in a maximal blastogenic response similar to that seen after adding α MM at 24 hr. Our data indicate that the apparent commitment of cells to DNA replication is longer than the 24 hr reported by many investigators.

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FIGURE LEGENDS

Figure 1. Stimulation induced by WGA and effects of GlcNAc. ^3H -Thymidine incorporation in WGA-stimulated peripheral blood lymphocytes from 6 normal individuals [A-F] with (o) and without (●) GlcNAc addition at 24 hr, as a function of WGA concentrations.

Figure 2. Late response to WGA and effects of GlcNAc. ^3H -Thymidine incorporation at 120-140 hr of WGA-treated lymphocytes with (o) and without (●) GlcNAc addition at 24 hr.

Figure 3. Effect of preincubation of cells on response to WGA. Cells were treated with WGA after preincubation for 24 hr at: [1] 0° C with (o) and without (●) GlcNAc addition at 24 hr; and [2] 37° C with (Δ) and without (▲) GlcNAc addition at 24 hr. Results of 4 experiments in 4 normals.

Figure 4. Stimulation by Con A: Effects of preincubation and αMM. ^3H -Thymidine incorporation of cells treated directly with Con A [direct] or treated after 24 hr preincubation at 37° C [preincubated] with (o) and without (●) αMM addition at 24 hr after Con A treatment. Results of 3 experiments in 3 normal individuals [A-C]. Comparable direct and preincubated experiments were done on the same preparation of lymphocytes.

Figure 5. Kinetics of cell response to Con A: Effect of preincubation. Kinetics of the preincubation effect in cells treated directly (●) or preincubated for 24 hr (○) prior to stimulation with 1 µg/ml Con A. Replicate cultures were pulsed with ³H-thymidine for 20 hr at varying times after Con A addition.

Figure 6. Response of cells to Con A: Effect of QMM addition prior to 24 hr. QMM was added to replicate cultures and compared with the response of cells to Con A without QMM addition (●). QMM was added prior to Con A (■), after 1 hr incubation of cells with Con A at 0° C (□) or 37° C (Δ), and after 4 hr incubation of cells with Con A at 37° C (○).

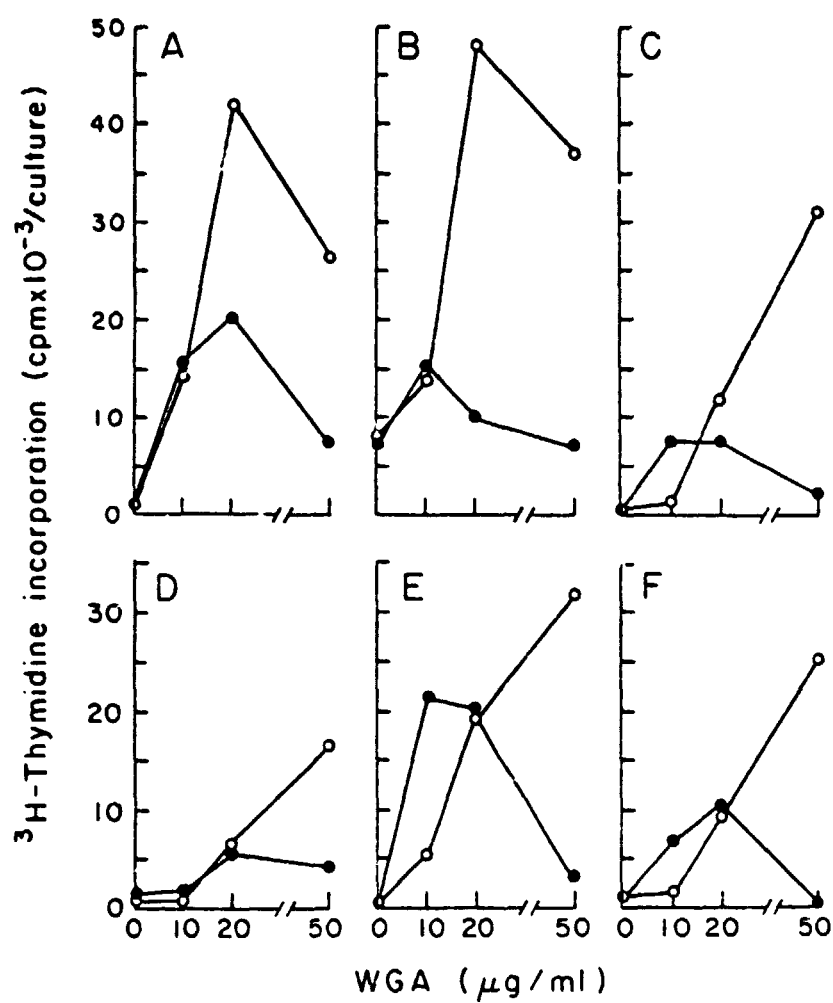


Figure 1

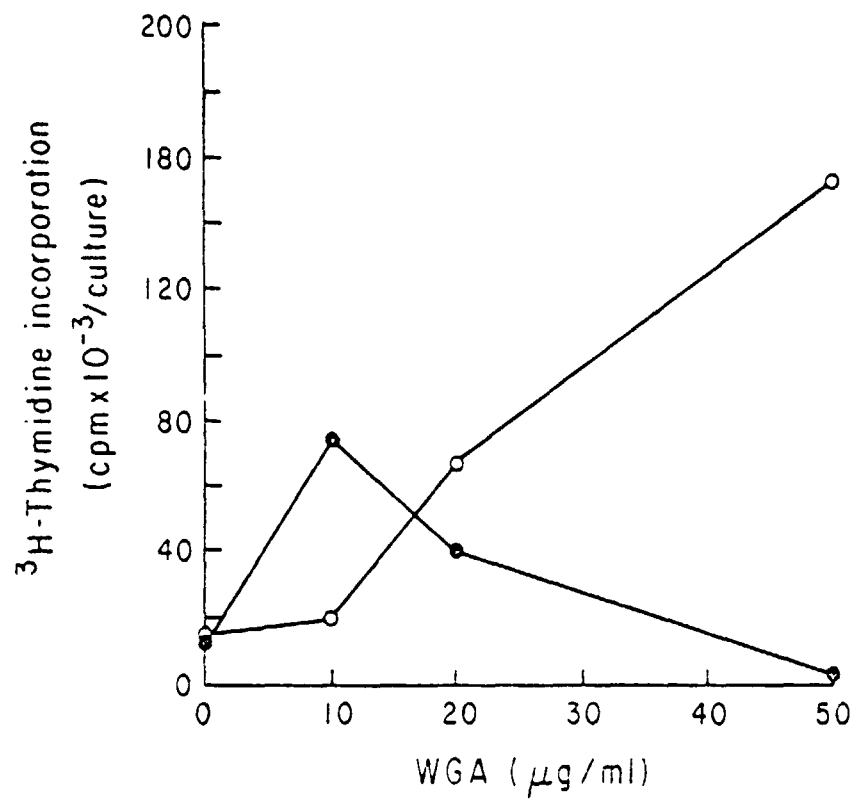


Figure 2

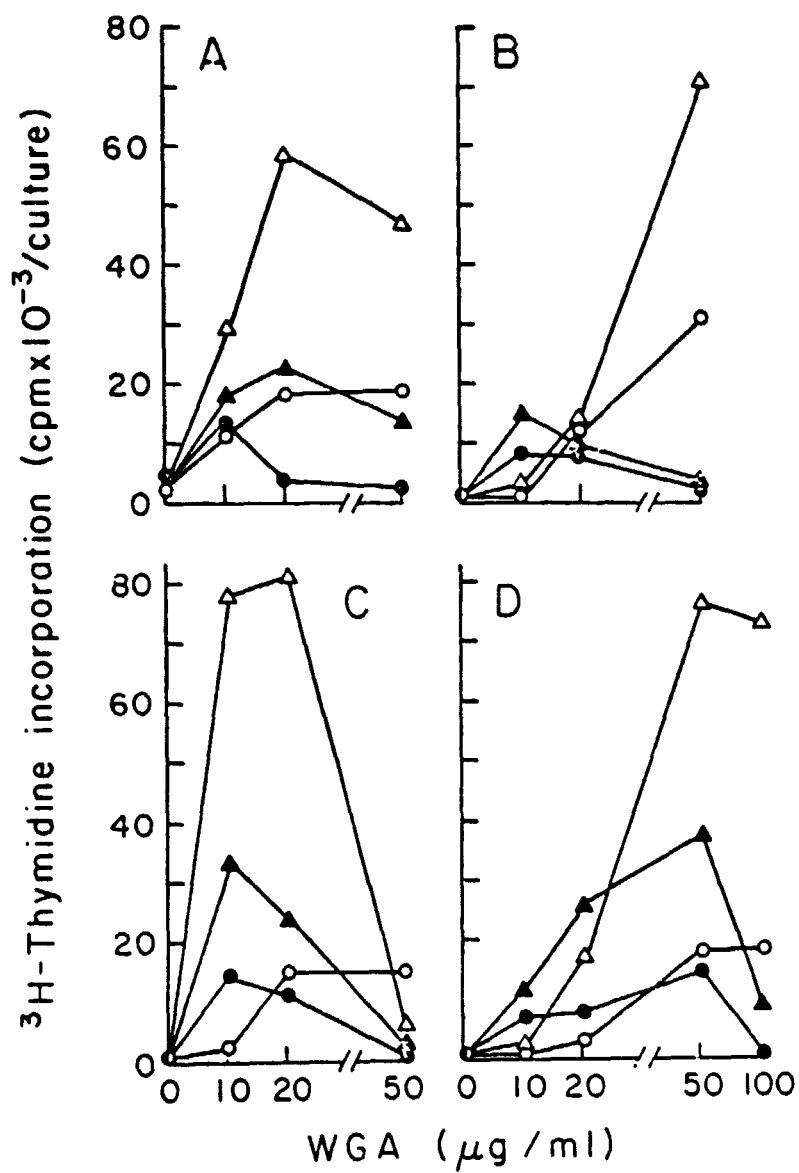


Figure 3

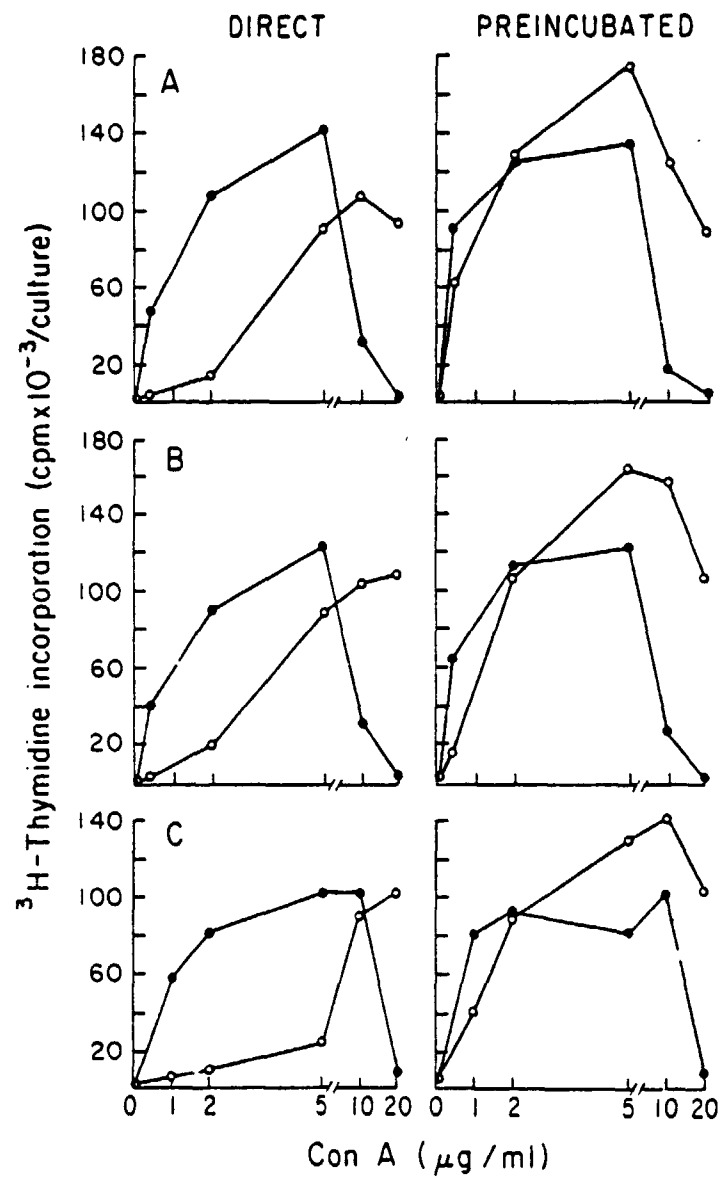


Figure 4

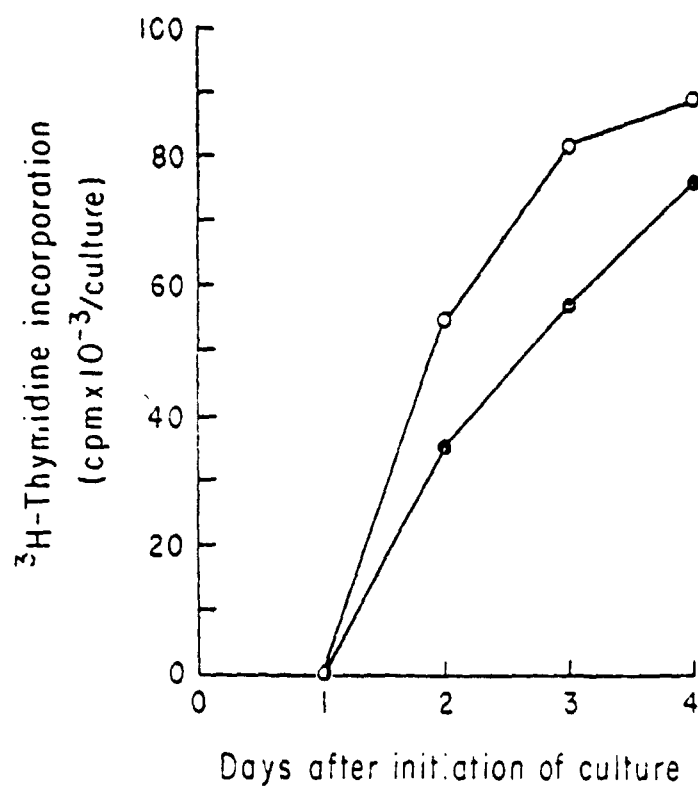


Figure 5

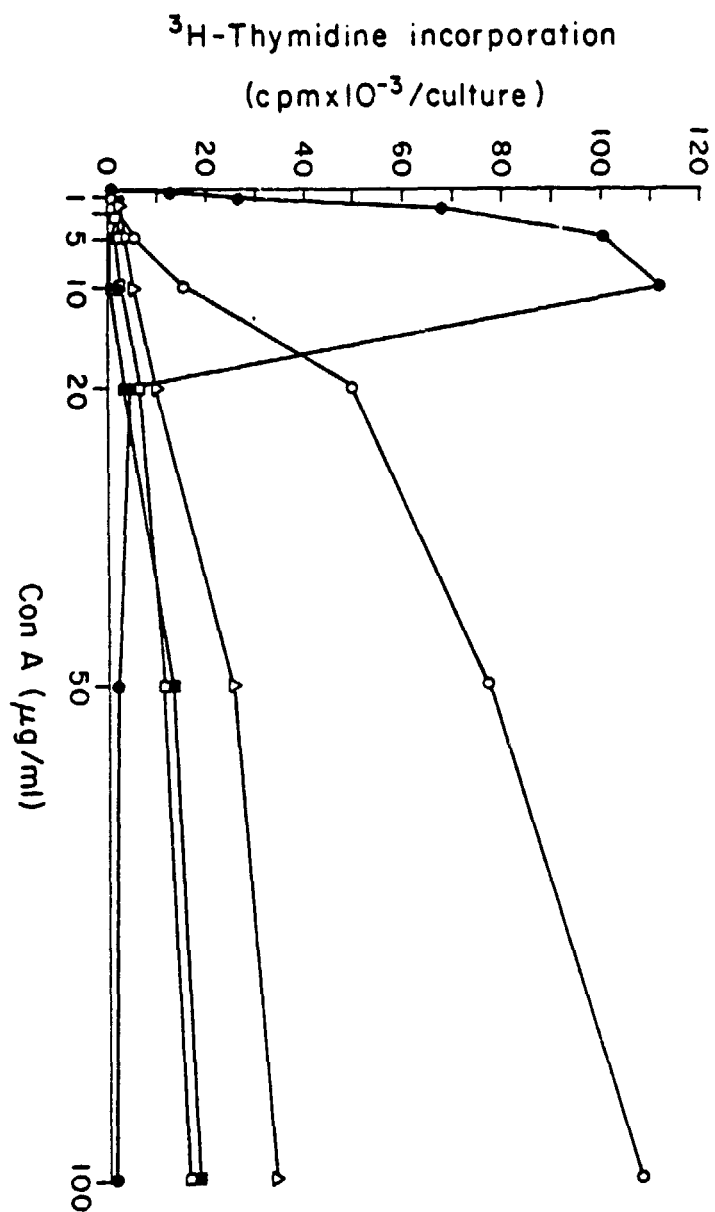


Figure 6